EXAMINING THE FUNCTIONAL ROLES OF AQUAPORINS AND UREA TRANSPORTERS IN THE MOVEMENT OF UREA ACROSS THE RUMINAL EPITHELIUM

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ABSTRACT

Ussing chamber studies of ruminal epithelial tissue have provided insight into the mechanisms that regulate serosal-to-mucosal urea transport. Of these mechanisms, urea transport (UT-B) proteins have been shown to facilitate urea movement across the ruminal epithelium; however, other mechanisms may be involved as well because inhibiting UT-B does not completely eliminate urea transport. Of the aquaporins (AQP), which are a family of membrane-spanning proteins that are predominantly involved in the movement of water, AQP-3, -7, and -10 are also permeable to urea, but it is not clear if they contribute to urea transport across the ruminal epithelium. My objectives were to determine the relative functional roles of UT- and AQP-mediated serosal to mucosal urea flux ($J_{sm\text{-}urea}$) in response to changes in dietary carbohydrate fermentability, as well as ruminal ammonia and blood urea concentrations.

The objectives of the Chapter 2 studies were: 1) to evaluate if there are differences in the magnitude of serosal-to-mucosal urea transfer in ruminal epithelium obtained from the caudal-dorsal or ventral sacs; 2) to determine the optimum mucosal buffer pH for maximal urea transport across the bovine ruminal epithelium; 3) to determine the time that is required for steady-state isotope equilibration with bovine ruminal epithelium; and 4) to determine if $NiCl_2$ and $HgCl_2$ are suitable inhibitors of aquaporin-mediated urea transport in bovine ruminal epithelium. Steady-state $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ fluxes were observed by 45 min following isotopic additions to the serosal buffer. Epithelia collected from the caudal-dorsal sac had higher $J_{sm\text{-}urea}$ ($P = 0.03$) and lower $J_{sm\text{-}mannitol}$ ($P < 0.01$) than that collected from the ventral sac. Reducing mucosal buffer pH from 7.0 to 5.2 increased $J_{sm\text{-}urea}$ quadratically, where $J_{sm\text{-}urea}$ increased from pH 7.0 to 6.4 and thereafter decreased ($P = 0.01$). Both $HgCl_2$ and $NiCl_2$ inhibited $J_{sm\text{-}urea}$ ($P < 0.01$); however, the addition of $HgCl_2$ increased Tissue Conductance (G_t) when compared to $NiCl_2$.

The objectives of Chapter 3were to determine: 1) the functional roles of AQP and UT-B in the serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) across rumen epithelium; and 2) whether functional adaptation occurred in response to increased diet fermentability. Serosal addition of phloretin. The addition of phloretin or $NiCl_2$ reduced the $J_{sm\text{-}urea}$ from 116.5 to 54.0 and 89.5 $nmol \cdot cm^{-2} \cdot h^{-1}$, respectively across all dietary treatments. When both inhibitors were added simultaneously, $J_{sm\text{-}urea}$ was further reduced to 36.8 $nmol \cdot cm^{-2} \cdot h^{-1}$. Phloretin-sensitive and $NiCl_2$-sensitive $J_{sm\text{-}urea}$

were not affected by diet. The $J_{sm\text{-}urea}$ tended to increase linearly as the duration of adaptation to MGD increased, with the lowest $J_{sm\text{-}urea}$ being observed in animals fed CON (107.7 nmol·cm^{-2}·h^{-1}) and the highest for those fed the MGD for 21 d (144.2 nmol·cm^{-2}·h^{-1}). Phloretin-insensitive $J_{sm\text{-}urea}$ tended to increase linearly as the duration of adaptation to moderate grain diet increased, whereas there was a tendency for $NiCl_2$-insensitive $J_{sm\text{-}urea}$ to be affected by diet. Gene transcript abundance for AQP-3 and UT-B in ruminal epithelium increased linearly as the duration of MGD adaptation increased. For AQP-7 and AQP-10, gene transcript abundance in animals that were fed the MGD was greater when compared to CON animals.

The objective of Chapter 4 was to determine the effect of an acute dose of NH_3 on total and aquaporin (AQP)-mediated urea flux across the ruminal epithelium in Angus cross bulls and Plains bison bulls. Ruminal NH_3 was not affected by species (P = 0.60) or diet (P = 0.27) while PUN tended (P = 0.055) to be greater for BIS (12.5 mg/dL) than BOV (10.8 mg/dL), but was not affected by diet (P = 0.22). The $J_{sm\text{-}urea}$ tended to decrease with addition of $NiCl_2$ (P = 0.065), while mucosal NH_3 had no effect on $J_{sm\text{-}urea}$ (P = 0.41). $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were not affected by species (P = 0.41) or dietary treatment (P = 0.29).

I evaluated the effects of mucosal NH_3 and serosal urea concentrations on total and phloretin sensitive $J_{sm\text{-}urea\ in}$ Chapter 5. High Ammonia (HA) tended to inhibit total $J_{sm\text{-}urea}$ with HU, but there was no effect of either NH_3 concentration on total $J_{sm\text{-}urea}$ with Low Urea (LU; interaction, P = 0.055). Addition of phloretin in the presence of serosal urea or mucosal NH_3 had no effect on $J_{sm\text{-}urea.}$ The $J_{sm\text{-}mannitol}$ was not affected by serosal urea (P = 0.86) or mucosal NH_3 (P = 0.22) concentration. The $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ tended to be positively correlated for HA (R^2 = 0.301, P = 0.08), but not Low Ammonia (LA; R^2 = 0.027, P = 0.70) in combination with LU. The same pattern was observed with High Urea (HU) treatments where $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ tended to be positively correlated for HA (R^2 = 0.329, P = 0.08), but not LA (R^2 = 0.111, P = 0.32). This research provides evidence that both AQP and UT-B play an important role in the $J_{sm\text{-}urea.}$ In the future, additional research will be required to determine the mechanisms involved in NH_3 inhibition of $J_{sm\text{-}urea}$, as this key step is critical in the role of urea-N recycling in ruminants.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

AQP	Aquaporin
ATP	Adenosine Triphosphate
BHBA	β-Hydroxybutyrate
BIS	Bison
BOV	Bovine
CP	Crude Protein
CON	Control
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
DMI	Dry Matter Intake
EDTA	Ethylenediaminetetracetic acid
GIT	Gastro intestinal tract
G_t	Tissue conductance
HA	High ammonia treatment
HU	High urea treatment
I_{sc}	Short-circuit current
$J_{ms\text{-}urea}$	Mucosal to serosal flux of urea
$J_{sm\text{-}urea}$	Serosal to mucosal flux of urea
$J_{sm\text{-}mannitol}$	Serosal to mucosal flux of urea
LA	Low ammonia treatment
LU	Low urea treatment

N	Nitrogen
NHE	Sodium hydrogen exchanger
NEFA	Non-esterified fatty acid
SCFA	Short-chain fatty acid
UNS	Urea-N salvage
UT	Urea transporter
UT-A	Urea transporter - A
UT-B	Urea transporter -B
VFA	Volatile fatty acid
MGD	Moderate grain diet

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

It is widely accepted that the ruminant gastro-intestinal tract plays a major role in metabolism of energy and protein. Researchers have noted that the rumen plays a particularly important role in the absorption of (SCFA and ammonia, while secreting significant amounts of urea which provides N for microbial protein synthesis (Lapierre and Lobley, 2001). Feed is fermented by the ruminal microorganisms to produce short chain fatty acids (SCFA), as well as microbial protein; however, excess N produced during the fermentation process is absorbed as ammonia-N and converted to urea in the liver (Reynolds and Kristensen, 2008). This urea can then, in turn, either be excreted through urine, feces and milk, or be recycled back to the rumen where it has the opportunity to be used by the ruminal bacteria for microbial protein synthesis. Although many mammalian species have the ability to recycle urea back across the gastro-intestinal tract for microbial use, this mechanism is of great importance to ruminants (Fuller and Reeds, 1998). This is because, in ruminants, microbial protein provides the majority of the metabolizable protein supply to the small intestine, accounting for 50 to 80% of total metabolizable protein (NRC, 2001). However, on first pass, a significant portion of the protein fermented by the ruminal microorganisms is used for energetic purposes, releasing the free N in the form of ammonia which is then absorbed by the ruminal epithelium. This ammonia-N can represent approximately half of the absorbed N and can make up 50% of the total ammonia-N flow to the liver, where it is detoxified to urea (Lapierre and Lobley, 2001). Typically in ruminants, urea release in the liver accounts for 65% of total N intake (Reynolds and Kristensen, 2008). This inefficiency at capturing dietary N upon first pass has significant limitations for ruminant survival in habitats with low N availability. If it were not for their ability to recycle the urea-N synthesized in the liver back to the rumen where ruminal microorganisms can use it as a source of N for microbial protein synthesis, ruminants would not be able to survive on low N diets. This mechanism, which is termed urea recycling or urea-N salvaging (UNS) (Lapierre and Lobley, 2001), has allowed ruminants to survive in environments that often offer little available protein N through grazing. As well, understanding this mechanism has provided researchers and nutritionists with a significant opportunity to manipulate protein nutrition, thereby increasing N efficiency in commercial livestock production. This N returning to the ruminal microorganisms is returned via two main pathways, through salivary secretions or through direct transport across

the ruminal epithelium, both of which vary with dietary roughage inclusion such as grass hay due to the potential increase in rumination and salivary flow (Lapierre and Lobley, 2001).

Although it was once thought that urea can passively diffuse across membranes, it is now widely accepted that there are in fact protein-mediated mechanisms that are responsible for the secretion of urea into the rumen for breakdown and use by the ruminal microorganisms (Bach et al., 2005). Although passive diffusion of urea can account for a significant proportion of the total urea transported across the ruminal epithelium, transcellular transport is arguably of greater importance (Allen, 1997). Thus, the focus of this review of the literature is to examine the mechanisms involved in urea recycling to the GIT, the impact of diet on urea transport across the ruminal epithelium, as well as the structure and function of specific mechanisms involved in both the paracellular and trans-cellular movement of urea across the ruminal epithelium.

1.2 Importance of urea recycling

Urea recycling is an important mechanism that has allowed ruminants to survive on low quality diets. Multiple studies have revealed that ruminants are inefficient at utilizing digestible N, compared to monogastrics (Lapierre and Lobley, 2001). Using stable isotopes, researchers have noted that in some cases digestible N intake is less than the amount of urea-N synthesized in the liver (Lapierre and Lobley, 2001). Endogenous protein losses through cell turnover as well as enzyme production can account for a significant difference between a low digestible N intake and higher levels of urea-N synthesis in the liver; much of this is due to the recycling of urea-N back to the digestive tract, particularly when ruminants are fed low quality diets. This mechanism is particularly important for the survival of ruminants as the percentage of dietary N intake compared to urea-N returned to the gut has been shown to be as low as 15% in cats (Russell et al., 2000) where for ruminants the urea-N recycling has been documented to range from 61 to 79% (Archibeque et al., 2000; Lobley et al., 2000). This urea-N can be utilized for microbial protein synthesis and is therefore important N source for the rumen microbes (Bach et al., 2005). When ruminal microbes flow out of the rumen to the small intestine, they are the most important component of metabolizable protein thus supplying essential amino acids for absorption by the host animal (Clark et al., 1992). Thus, the mechanism of UNS is of importance to ruminants, as well as presenting itself as a possibility to increase efficiencies in ruminant production systems.

Dietary manipulation to improve ruminant nitrogen use has been investigated for many years. Zuntz first proposed in 1891 that ruminal bacteria have a significant role in the utilization of non-protein N in ruminants (Huntington and Archibeque, 1999). More recently, the primary focus for dietary manipulation is to increase either the efficiency of N use, in an effort to increase productivity while reducing both the environmental impact of excessive N excretion as well as reducing the labour involved in modern day livestock production systems. Much of the focus in lactating dairy cattle has been on synchronizing the rates of carbohydrate and N fermentation in the ruminal ecosystem. Providing high-producing dairy cattle with adequate protein and energy is crucial to allow them to attain high levels of milk production; however, feeding excess N to dairy cattle is energy-inefficient because of the energy costs of ammonia detoxification through ureagenesis. Huntington and Archibeque (1999) noted in their review that 4 mol of ATP are used for every 1 mol of urea produced through the ornithine cycle, representing as much as 2.5 to 5% of whole body oxygen consumption in lactating dairy cattle (Lobley et al., 1996). Thus, decreasing the dietary protein for high producing ruminants can be energetically efficient, or a missed opportunity for many nutritionists.

While meeting the demands of the high producing dairy cow includes providing more digestible protein, this can be achieved by maximizing microbial protein production as opposed to feeding high levels of ruminally undegradable protein. Adding starch to a diet composed of a degradable protein supplement and low quality hay decreased ruminal NH_3 along with fiber digestion and forage intake; however, the flow of amino acids to the duodenum was increased (Olson et al., 1999). Hristov et al. (2005) found similar results when they compared adding carbohydrates differing in the rate of fermentability to diets consisting of alfalfa hay and a source of ruminal undegradable protein. They noted that, along with a reduction in ruminal NH_3, that microbial N flow to the duodenum was increased with increasing dietary carbohydrate fermentability. Along with increasing diet fermentability through cereal starch addition as a method of increasing microbial production thereby reducing ruminal NH_3, providing ruminants with a source of dietary sugar has been shown to further increase N efficiency. Broderick et al. (2008) noted that when lactating Holsteins were fed diets containing either 0, 2.5, 5.0 or 7.5 (% of DM) sucrose as a replacement for dietary starch, that ruminal NH_3 was decreased linearly as dietary sucrose inclusion was increased. As well, milk fat content and milk fat yield were both increased by replacing dietary starch with sucrose. Similar results were reported by Chibisa et

al. (2015) where replacing dietary starch (barley or corn starch) with lactose decreased ruminal NH_3 as well as plasma urea nitrogen; however, no increase in milk production was observed in their study. Regardless of method of increasing diet fermentability, it remains clear that adding readily fermentable carbohydrates to ruminant diets can reduce ruminal NH_3, by increasing microbial protein synthesis.

Another management practice to maximize N efficiency in ruminants is to periodically limit digestible N intake. As opposed to synchronizing dietary protein and carbohydrate fermentation, this approach focusses on the dissociation in dietary synchronization, involving asynchrony periods of days as opposed to a few hours. In 1962, McIlvain and Shoop noted that when steers were grazing rangeland and supplemented with cottonseed meal in equal weekly amounts that there was no difference in average daily gain between steers that were fed supplemental protein on a daily, every third day or on a weekly basis. This has practical significance where animals fed oscillating high and low protein diets compared to a moderate protein diet, have higher N retention rates as well as excrete less urinary N (Doranalli et al., 2011). This has been shown to translate into greater growth rates in wethers (Cole et al., 1999; Cole et al., 2003; Archibeque et al., 2007; Doranalli et al., 2011) as well as in steers (Ludden et al., 2003). This increase in growth rates has been demonstrated to be due to the increase in urea recycled back to the rumen (Archibeque et al., 2007), in part due to a greater transfer of urea across the ruminal epithelium (Doranalli et al., 2011).

1.3 Ammonia production

Urea recycling in ruminants begins with the production of NH_3 in the rumen, leading to the absorption of NH_3 across the ruminal epithelium into the bloodstream. Depending on dietary and other conditions, ruminants can also absorb significant amounts of NH_3 from the lower digestive tract. Ammonia-N is produced through two pathways: 1) primarily through microbial breakdown of nitrogenous compounds such as proteins and amino acids; and 2) through microbial hydrolysis of urea that has been recycled back to the rumen (Abdoun et al., 2007). Early work by Bryant and Robinson (1962) demonstrated that 90% of the cultured ruminal bacterial species had the ability to use NH_3 as their sole source of N for sustaining microbial growth; however, once absorbed inside the microbial membranes, the available N used to can be used tosynthesize amino acids. Furthermore, amino acids derived through extracellular

degradation of dietary protein can be absorbed into the microbial cytosol. These amino acids are subjected to two fates depending on available carbohydrates (Bach et al., 2005). If carbohydrate energy sources are readily available, these amino acids can be used directly for incorporation into microbial protein (Mackie and White, 1990), while in energy-limiting environments where carbohydrate sources are scarce, amino acids will be deaminated leaving their carbon skeleton for fermentation producing SCFA (Tamminga, 1979).

Ruminal NH_3 concentrations often vary between dietary conditions and protein availability to the ruminal microorganisms (Firkins et al., 2007). Regardless of dietary protein concentration, a ruminal NH_3 concentration below 5 mg/dL (NRC, 2001) is thought to restrict microbial growth due to lack of available N. In addition to dietary protein intake, ruminal protein degradability, carbohydrate availability, as well as microbial populations present in the ruminal environment can greatly affect ruminal NH_3 concentration. Studies by Klusmeyer et al. (1990) noted that ruminal NH_3 concentration averaged 10.5 when animals were fed a diet containing 14.5% crude protein consisting mainly of soybean meal compared to 5.4 mg/dL when dairy cattle were fed a diet containing 14.5% crude protein consisting primarily of corn gluten meal as the major protein source,. The authors noted that reducing the ruminally degradable protein while increasing the ruminally undegradable protein through increased corn gluten meal supplementation led to a potential limitation in microbial protein yield, partly because of a limitation in NH_3 availability. However, Oba and Allen (2003) and Voelker and Allen (2003) noted ruminal NH_3 concentrations of 7.2 and 19.5 mg/dL with diets similar in protein (18% CP) but differing greatly in rumen starch and fiber fermentation rates. This is in agreement with Hristov et al. (2005), who fed dairy cows a basal diet supplemented with three sources of carbohydrate (glucose, corn starch or oat hulls) which varied in the rates and extents of ruminal starch degradability. They noted that as carbohydrate fermentability increased, ruminal NH_3 concentration decreased from 16.4 (oat hulls) to 8.5 mg/dL (glucose), resulting from an increase in the microbial use of peptides and amino acids for protein synthesis with glucose as the carbohydrate source as opposed to deamination and fermentation resulting in NH_3 production with oat hulls. As a result of this, blood urea nitrogen was also depressed on the highly-fermentable diet; microbial nitrogen flow from the rumen was highest on the glucose or highly fermentable diet compared to the slower fermenting corn starch or oat hull treatments. This synchronization of dietary protein and carbohydrate fermentability has been noted to maximize

microbial protein yield in lactating dairy cattle, providing a high quality protein source for milk protein synthesis (Huntington and Archibeque, 1999).

Although protozoa and fungi also have the ability to hydrolyze protein, bacterial N metabolism in the lower digestive tract accounts for the majority of the N sources in the ruminal environment (Hoover and Stokes, 1991) as well, ruminal bacterial N metabolism accounts for the largest portion of protein degradation in the ruminal environment (Bach et al., 2005). Kiran and Mutsvangwa (2009) examined the impact of ruminal protozoal population on bacteria through partial defaunation by feeding of linoleic acid-rich sunflower oil in lambs fed varying levels of dietary protein. They noted that partial defaunation reduced ruminal NH_3 concentrations as well as improved total nitrogen retention when compared to faunated lambs. The authors reported that removing of the ruminal protozoal populations resulted in a reduction in the predation of ruminal bacteria as well as having decreased competition for rumen available starch thereby increasing the urea-N recycled back to the GIT and resulting in an improved efficiency of N usage.

1.4 Ammonia absorption

The excess NH_3 produced at a rate exceeding the N needs of the ruminal microbes is absorbed across the ruminal epithelium for detoxification in the liver. As ruminal NH_3 concentrations increase, the rate at which NH_3 diffuses across the ruminal epithelium also increases (Hogan, 1961; Remond et al., 1993). It has also been reported that ruminants fed high protein diets for prolonged periods of time, resulting in high ruminal NH_3 concentrations, will have higher rates of ruminal NH_3 absorption (Abdoun et al., 2003). However with a pKa of 9.25 (Leng and Nolan, 1984), 99% of the NH_3 will be in the ionized form of NH_4^+ at a pH between 6 and 7 which is more typical of the ruminal environment. Charged compounds diffuse poorly across lipid cell membranes, such that NH_3 is approximately 175 times more permeable to the ruminal epithelium than NH_4^+ (Bödeker et al., 1990); however, when intra ruminal pH is lowered, NH_4^+ permeability across the ruminal epithelium is depressed such that NH_3 transfer to the liver remains stable despite increases in ruminal NH_3 concentration (Hogan, 1961; Bödeker et al., 1990).

Due to the abundance of NH_4^+ at typical ruminal pH levels, researchers considered the possibility of channels or carriers that aid in the transport of NH_4^+ across the epithelial membrane. Bödeker and Kemkowski (1996) noted that ruminal epithelium under Ussing

chamber incubations had a short circuit current (I_{sc}) increase when quinidine, an inhibitor for K^+ channels, was added to the mucosal buffer in combination with NH_4Cl and KCl, but was not elevated when choline chloride was added. They later clamped the transepithelial potential difference at +25mV, and noted that NH_4^+ transport, as well as K^+ transport, was markedly reduced in the presence of quinidine, leading these researchers to conclude that it is likely that NH_4^+ was transported across the lipid membrane via K^+ channels. This was later confirmed by Abdoun et al. (2005), who noted that increasing luminal NH_3 concentrations through luminal addition of NH_4Cl increased the net flux of sodium across the ruminal epithelium ($J_{net\text{-}Na}$) transport across the isolated ovine ruminal epithelium at a luminal pH of 6.4, whereas $J_{net\text{-}Na}$ transport was unaffected by luminal NH_4Cl concentration at a luminal pH of 6.9. They concluded that Na transport across the ruminal epithelium is inhibited by NH_3 entering the cytosol; however, NH_4^+ entering through K^+ channels has a stimulatory effect on net Na^+ flux. Lu et al. (2014) proposed that the mechanism of NH_3 stimulating Sodium-Hydrogen exchanger (NHE) activity is similar to the mechanism of SCFA absorption. At a physiological pH (~ 6.4), ammonia will be absorbed in the protonated state (NH_4^+). Once in the cytosol (pH ~ 7), NH_4^+ will be dissociated to NH_3 and H^+, resulting in a reduction in intracellular pH. As a mechanism to maintain cytosolic pH, NHE activity is up-regulated thereby removing H^+ ions from the cell back to the ruminal fluid. The resulting NH_3 is then able to dissociate through to the portal blood, exiting the cytosol via K^+ channels. This was confirmed by Lu et al. (2014), who observed a depression in $J_{net\text{-}urea}$ and an increase in $J_{net\text{-}Na}$ with increasing luminal NH_3 concentrations. This increase in NH_3 absorption can be detected via increases in $J_{net\text{-}Na}$ as well as increases in I_{SC} across the isolated ruminal epithelium (Lu et al. 2014).

1.5 Urea synthesis in the liver

Ammonia that is absorbed from the rumen is transported via the portal vein to the liver for detoxification. In the periportal hepatocytes, NH_3 is detoxified into urea via the ornithine cycle, a mechanism termed ‘low capacity and high affinity’ by Haussinger et al. (1992). The ornithine cycle begins in the mitochondria with the condensation of NH_3 with bicarbonate to form carbamoyl phosphate, a reaction that is catalyzed by carbamoyl phosphate synthetase (Meijer et al., 1990), where the first N needed for the formation of urea. The second step involves the reaction of carbamoyl-phosphate with ornithine, catalyzed by ornithine transcarbomoylase, to form citrulline. This newly formed citrulline is then translocated out of

the mitochondria to the cytosol where it is condensed with aspartate to form arginosuccinate, a reaction catalyzed by arginosuccinate synthase. Fumarate is then removed from the arginosuccinate through the enzyme arginosuccinate lyase; fumarate is then made available to enter the tricarboxylic acid cycle and serve as a substrate for aspartate production. From this reaction, arginine is left available for degradation by arginase to produce ornithine as well as urea. Ornithine is then available for further participation in ureagenesis, with urea being released into the bloodstream.

During periods when excessive quantities of NH_3 are entering the periportal system, when at a rate exceeding the ornithine cycle's capacity of ureagenesis, the spill-over of NH_3 is dealt with in the perivenus hepatocytes. Here, there is a 'high affinity, low capacity' system (Haussinger et al. 1992) in which the high glutamine synthetase activity is able to sequester free NH_3, catalyzing the condensation of glutamate and NH_3 to form glutamine, which is a metabolically safe compound. Glutamine is released into the bloodstream. Upon glutamine re-entering the periportal hepatocytes, glutamine dehydrogenase deaminates glutamine to glutamate, leaving the newly freed NH_3 to be used in the ornithine cycle for ureagenesis. Urea that is produced in the liver is released into the bloodstream. Although a significant portion of blood urea is excreted in the urine, ruminants have the ability to recycle 40 to 80% of their endogenously-produced urea to the digestive tract via salivary secretion (Huntington, 1989) or across the epithelia lining the digestive tract through diffusion (Houpt and Houpt, 1968) or protein-mediated transport (Ritzhaupt et al., 1997).

1.6 Salivary secretion of urea

Salivary secretion of urea is an important mechanism in urea recycling in ruminants, and the magnitude of urea recycling through this mechanism can vary widely depending on factors such as dietary forage:concentrate ratio and blood urea concentration (Lapierre and Lobley, 2001). This salivary urea contribution has been noted to total 17% when steers were fed a high concentrate diet (Guerino et al., 1991), to 36% when animals were fed a forage-based diet (Taniguchi et al., 1995). Lapierre and Lobely (2001) reported that when dairy cattle were fed diets where 50% of the forage portion was grass hay, saliva inflow accounted for 47% of the total urea inflow to the rumen, thus demonstrating the significance of this mechanism. Sunny et al. (2011) noted that there was a linear relationship between plasma urea concentration and urea

recycled to the GIT in lambs. This is in agreement with Lewis (1957) where he noted that an increase in blood urea concentration increased salivary urea content.

1.7. Methods of measuring urea kinetics

1.7.1 Whole body urea kinetics

One of the methods used to study whole body urea kinetics in ruminants has been widely used by researchers attempting to determine the changes in nitrogen usage under different dietary treatments. Initially the use of this technique was simply for the estimation of urea entry rate (UER), mainly from the synthesis of urea in the liver, as well as a measure of urinary urea excretion (Lapierre and Lobley, 2001). However, the use of [^{15}N]urea has allowed researchers to quantify the rates of ureagenesis as well as quantify the amounts transferred to the whole digestive tract, or through specific sites of sampling, to the rumen (Chibisa et al., 2015). This approach was developed through a technique used to determine urea-N movement in humans (McClelland and Jackson, 1996). This adaptation of the original technique involves the infusion of [^{15}N^{15}N] urea, followed by analyzing for three forms of urea, [^{15}N^{15}N], [^{15}N^{14}N], and [^{14}N^{14}N] that are excreted from the animal. From these calculations, gut entry rate of urea (GER) can be calculated through the difference between the isotopic dilution of [^{15}N^{15}N]urea and the urinary urea excretion. Fates of urea can be monitored through measuring the amount of [^{15}N] appearance in the feces as well as the quantity of the [^{15}N^{14}N]urea excreted in the urine. Formation of [^{15}N^{14}N]urea results from the degradation of the infused or injected [^{15}N^{15}N]urea by the rumen microbes to ammonia. Some of this ammonia is incorporated into microbial protein, while a portion of this is absorbed across the rumen wall, and detoxified to urea in the liver, thus the proportion of [^{15}N^{14}N]urea recovered can be used to calculate urea recycled back to the rumen microbes for microbial protein synthesis. Calculating the difference between the total urea-N entry and the amount catabolized results in the estimated urea-N used for anabolic purposes such as microbial protein synthesis. Although this technique is widely accepted as a means of determining the recycled urea transported to the rumen (Chibisa et al., 2015), calculating the proportion of urea that is transported using specific mechanisms such as paracellular transport or facilitative transport mechanisms, is not possible using this method. Thus examining other techniques was needed for the purposes of this thesis.

1.7.2 Ussing Chamber

Developed by Hans Ussing in the 1950s to study electrolyte transport across frog skin, the circulating Ussing chamber technique is a highly adaptable method for measuring absorption across a variety of tissues (Li et al., 2004). The circulating Ussing chamber is depicted in Figure 1.1. This chamber system consists of a pair of columns that contain the experimental buffer solution; the solution must contain all of the necessary nutrients for tissue metabolism to prevent premature tissue death (Li et al., 2004). This tubing allows for the buffer solution to be gassed during the experiment with gas combinations similar to those of the washed reticulorumen, to both oxygenate the tissue as well as mix the solution in either column (Bricker et al., 1963). The tubing is connected to the Ussing chamber, a cylinder divided into two equal halves where the tissue sample is mounted between the Ussing chamber halves as shown in Figure 1. Attached to both halves of the Ussing chamber, are two electrodes. One electrode from each side is connected to a current supply while the other electrodes are connected to a voltmeter. The voltmeter measures the action potential across the tissue and can be manipulated by the addition of an electrical current across the epithelium. Voltage measurements across the epithelium allow researchers to quantify electrolyte transport of the specific tissue (Bricker et al., 1963).

Tissue samples must be collected from freshly euthanized animals, and quickly mounted onto the chamber. To measure tissue viability, researchers are able to do one of two things. They are able to 'clamp' or hold the voltage constant across the tissue, thereby applying a current across the tissue. Using Ohm's law, the tissue resistance can be calculated; as tissue resistance begins to decline this indicates that the integrity of the tissue is decreasing. The second method for measuring tissue resistance is to use a current clamp, where a constant current is applied across the tissue and the voltage is measured. Again Ohm's law can be used to calculate the tissue resistance (Li et al., 2004).

Abdoun et al., (2010) set out to measure the bi-directional movement of urea across the rumen epithelium of sheep, using the circulating Ussing chamber technique. They collected rumen epithelial tissue from the ventral sack of freshly slaughtered lambs fed common diets. Tissue was washed in a buffer solution and the mucosal layer was stripped from the muscle layer. The tissue was then placed in a transport buffer solution that had been gassed with 95% O_2 and 5% CO_2 which is in agreement with Dorinalli et al., (2011). Upon arrival at the lab,

tissue samples were mounted between the two halves of the chamber and tissue resistance was measured. Experimental solutions containing electrolytes, volatile fatty acids and antibiotics were added to the each column and continuously gassed; one column on each chamber was dedicated to mimic the rumen conditions while the other column representing the basal membrane conditions (Abdoun et al., 2010). To measure the flux of urea across the epithelium, C^{14} labeled urea was added to the 'hot' column and its appearance was measured in the adjacent column.

1.7 Mechanisms of urea transport across the ruminal epithelium

1.7.1 Simple passive diffusion

The transport of urea across the ruminal epithelium was thought to be simply by passive diffusion across the ruminal wall as it is a small non-charged molecule, moving down a concentration gradient from the blood into the ruminal fluid (Houpt and Houpt, 1968). In the rumen, recycled urea is immediately broken down to NH_3 by bacterial urease (Lapierre and Lobley, 2001). This breakdown of urea into NH_3 creates the concentration gradient to drive the passive diffusion of urea across the ruminal epithelium (Wallace et al., 1979; Rémond et al., 1996).

1.7.2 Urea transport proteins

In the past, urea was thought to passively diffuse across the ruminal epithelia, due to osmotic effects of a high blood urea concentration combined with a low ruminal urea concentration (Sunny et al., 2007). However, due to the poor lipid solubility of urea (Galluci et al., 1971), researchers began to examine the membrane spanning proteins that had been demonstrated to facilitate urea transport in other tissues, in particular the kidney (Sands, 2003). These urea transporter (UT) proteins , ranging from 43 to 117 kDa in size (Sands, 2003), have been discovered to move urea across epithelial layers in human and rat kidney, as well as human colon via facilitative transport (Collins et al., 2010). Similarly in ruminants, UT-A has been discovered in ovine small intestinal epithelia (Marini and Van Amburgh, 2003), while UT-B has been discovered in bovine and ovine rumen epithelial tissue (Ritzhaupt et al., 1997; Marini and Van Amburgh, 2003). Using the ex vivo Ussing chamber technique, several research groups (Stewart et al., 2005; Abdoun et al., 2009; Kiran et al., 2011, Lu et al. 2015a) demonstrated that phloretin, a known inhibitor of facilitative urea transport via UT proteins, reduced trans-

epithelial flux of urea in isolated ruminal epithelia, thus providing evidence that UT play a functional role in trans-epithelial urea transfer in ruminants.

Dietary regulation of UT proteins in the ruminal epithelium has yet to be fully elucidated. Marini et al. (2004) reported that when lambs were fed diets with increasing crude protein of 9.8, 17.8, and 25.3%, lambs fed the high CP diets had lower rates of urea reabsorption into the GIT. The authors also noted that lambs fed lower CP diets had higher rates of bacterial urease activity, typically associated with epimural bacteria. These results indicate that lambs fed low CP diets were recycling and making use of urea-N more efficiently than lambs fed high CP diets. However, Marini et al.(2004) were unable to detect differences in UT-B abundance in the rumen, duodenum, ileum, cecum, liver, and kidney. They concluded that although their study demonstrated that there was significant ruminal adaptation to low nitrogen intakes, the increase in urea transfer to the GIT was not mediated through an increase in UT-B protein abundance. However, it should be noted that UT-B function in urea transport was not evaluated directly in this experiment. Similar findings were noted by Lu et al. (2014) where the presence of 1.25 *mM* NH_4Cl in cultured epithelial cells harvested from goats resulted in a significant reduction of UT-B mRNA abundance at a pH of 6.8; however, no difference was noted at a pH of 7.4, even though maximal UT-B expression was noted at a pH of 7.4, when in the presence of urea, not NH_4. They also noted that in ruminants fed diets high in ruminally fermentable non-fiber carbohydrates (NFC) as well as N, there was a greater abundance of mRNA for UT-B in ruminal epithelial tissue compared to that harvested from goats fed diets low in dietary NFC and N. Although the authors suggested that their findings fit with classic urea recycling theories and that UT-B plays a significant role in the transport of urea across the ruminal epithelium, other mechanisms may be involved. Critical evaluation of this data also suggests that, while UT-B may contribute to the transepithelial urea flux, the greater activity of UT-B at a ruminal pH of 6.8 and greater may imply that its overall contribution may be overestimated given that a pH of 6.8 and greater is not seen in high producing lactating dairy cattle.

UT protein expression has been shown to increase when ruminants are fed diets that contain large proportions of readily fermentable carbohydrates (Simmons et al., 2009). By increasing rapidly fermentable carbohydrates in ruminant diets, this results in more fermentable substrate for ruminal bacterial fermentation. When this is coupled with an adequate supply of

available N sources, the result is an increase in ruminal microbial growth (Huntington and Archibeque, 1999). This increase in microbial growth creates a strong osmotic gradient between ruminal and blood urea concentrations (Sunny et al., 2007). Simmons et al. (2009) noted that when steers were offered either silage- or concentrate-based rations differing in ruminal carbohydrate fermentability, there was an increase in the abundance of total UT-B proteins in the concentrate fed steers. They went on to examine how these UT-B proteins were distributed throughout the ruminal epithelium using immunohistochemistry. They concluded that, like other membrane spanning proteins such as NHE or Na^+/K^+-ATPase, the UT-B family of proteins were found predominantly in the strarum basale and stratum spinosum layers of the ruminal epithelium. This implies that UT-mediated urea transport across the ruminal epithelia is a highly regulated mechanism as the proteins are found in the metabolically active layers of the ruminal epithelium. This was also observed by Lu et al. (2015b) who reported that, in ruminal epithelial cells cultured from goats fed diets differing in dietary NFC, mRNA abundance of UT-B was greatest in cells cultured at a pH of 6.8 when compared to a pH of 7.4 in the presence of a 40 *mM* SCFA mixture. A ruminal pH of 6.8 would be more indicative of a more metabolically-active ruminal microbial population that can make anabolic use of any recycled urea-N. This is in agreement with current theories regarding urea recycling, where an increase in fermentable carbohydrates leads to a decrease in ruminally available nitrogen which, in turn, increases UT-B expression in an attempt to meet the increased demand of urea transport across the ruminal wall (Abdoun et al., 2010). Clearly, UT proteins play a role in the serosal to mucosal transport of urea across the ruminal epithelium.

1.7.3 Aquaporins

Aquaporins (AQP) are a large family of membrane proteins that are found in a variety of tissues including the kidney (Tchekneva et al., 2008) and the gastrointestinal tract (GIT) of humans and rodents (Ma and Verkman, 1999). These AQP can be divided into three subgroups: 1) the classical AQP which transport only water; 2) aquaglyceroporins that have the ability to transport small, uncharged molecules in addition to water; and 3) the unorthodox aquaporins that are still not fully understood (Tchekneva et al., 2008). In addition to water transport, aquaglyceroporins (AQP3, AQP7, AQP8, AQP9, and AQP11) have also been demonstrated to transport small, uncharged molecules like urea in non-ruminant species (Rojek et al., 2008). For example, in AQP11 knockout mice, the absence of this single AQP caused a 4.7-fold increase in

blood urea nitrogen when compared to wild-type mice (Tchekneva et al., 2008). When expressed in *Xenopus* oocytes, mouse AQP8 has been shown to increase urea permeability (Calamita et al., 1999). Although there has been significant research to determine the prevalence and functional roles of aquaporins in the kidneys and GIT of human and non-ruminant species, to my knowledge there is only one study (Røjen et al., 2011) demonstrating the presence of AQP in the GIT of ruminants. Røjen et al. (2011) observed an increase in mRNA and protein abundance of AQP8 in ruminal epithelia of dairy cows that were fed a high protein diet compared to those fed a low protein diet. Based on these observations, Rojen et al. (2011) concluded that AQP are not the primary mechanism responsible for facilitative transport of urea across the rumen wall; however, they tested only protein abundance and not protein function in their study.

1.8 Conclusions

Ruminants have survived on low quality forages for thousands of years, in part due to their ability to conserve N when environmental availability for dietary N is scarce. Under current ruminant production systems, when dietary protein is in abundance, excess NH_3 is converted to urea in the liver through the ornithine cycle. This urea can then be transported back to the GIT, through saliva secretions or transported across the epithelium, where it is used for microbial protein synthesis which is a significant protein source for ruminants. Researchers have demonstrated that UT proteins play a significant role in the trans-epithelial movement of urea; however, it is also clear that other proteins such as AQP may play a role in this movement of urea across the ruminal epithelium.

OVERALL HYPOTHESIS

My overall hypothesis was that urea transporters and AQP play functional roles in urea transport from blood into the rumen and potentially mediate known effects of factors such as the dietary level of ruminally-fermentable carbohydrate, ruminal environmental conditions (ruminal pH, ammonia), and plasma urea-N concentration on urea recycling.

OVERALL OBJECTIVES

To determine the relative functional roles of UT- and AQP-mediated urea flux in ruminal epithelia in response to changes in dietary carbohydrate fermentability, as well as ruminal ammonia and blood urea concentrations.

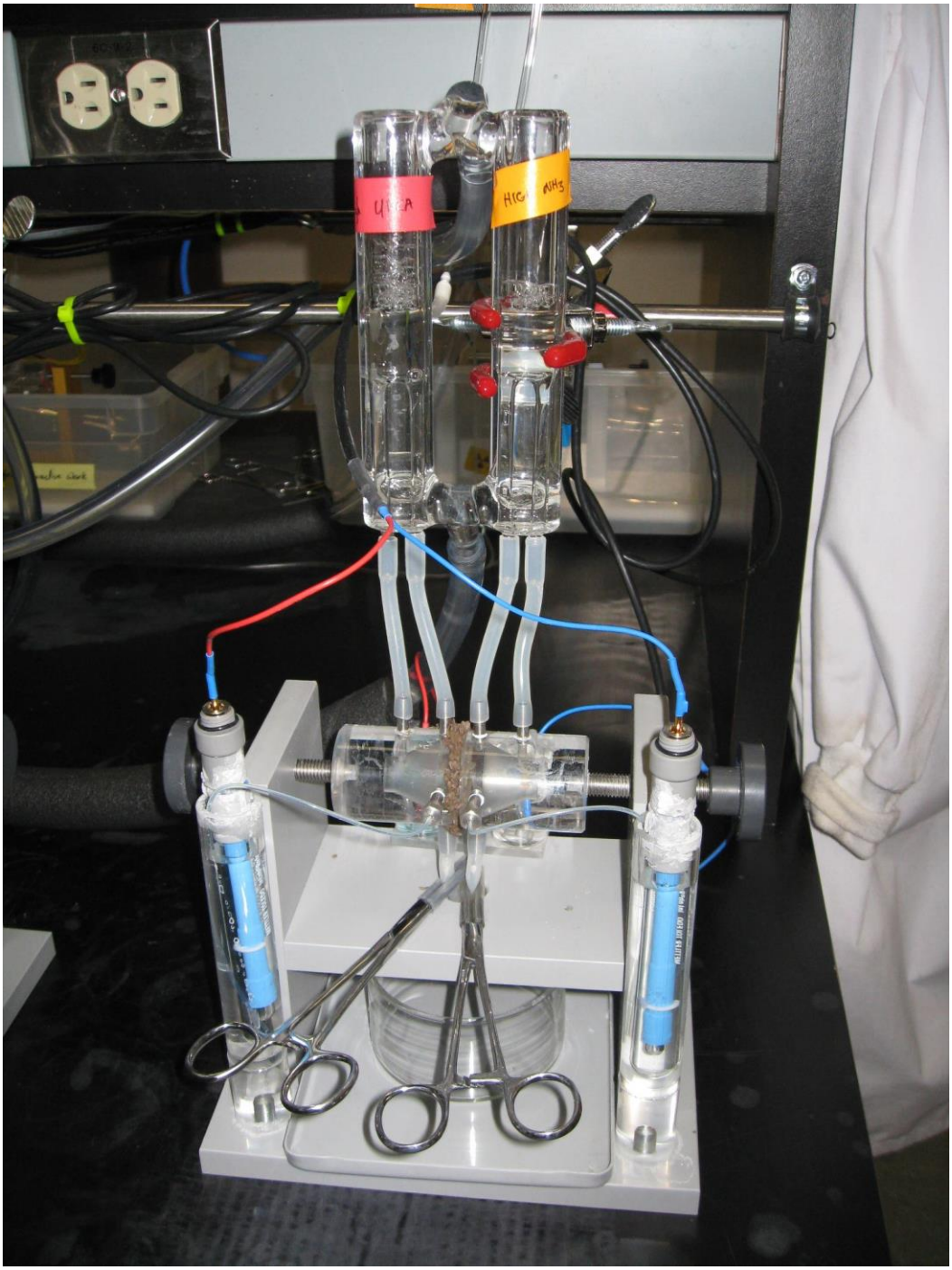

Figure 1.1. Ussing Chamber System incubating ruminal epithelial tissue. Mucosal buffers are contained on the right, with serosal buffer located on the left. Note: buffers contained in a glass, water jacketed device where buffers are continuously circulating via gas lift with 95% O2/5%CO2. Voltage electrodes are shown at in the forefront of the stand, while current electrodes are not visible.

CHAPTER 2. PRELIMINARY STUDIES TO VALIDATE THE EFFECTS OF INHIBITORS, ISOTOPE EQUILIBRATION, RUMINAL TISSUE LOCATION, AND MUCOSAL INCUBATION BUFFER pH.

2.1 Abstract

Ussing chamber studies of ruminal epithelial tissue have provided insight into the mechanisms that regulate serosal-to-mucosal urea transport; however, most studies have used ovine ruminal epithelium and the optimum conditions that are required for the incubation of bovine ruminal epithelium under Ussing chamber conditions are unknown. The objectives of the present studies were: 1) to evaluate if there are differences in the magnitude of serosal-to-mucosal urea transfer in ruminal epithelium obtained from the caudal-dorsal or ventral sacs; 2) to determine the optimum mucosal buffer pH for maximal urea transport across the bovine ruminal epithelium; 3) to determine the duration of time that is required for steady-state isotope equilibration with bovine ruminal epithelium; and 4) to determine if $NiCl_2$ and $HgCl_2$ are suitable inhibitors of aquaporin-mediated urea transport in bovine ruminal epithelium. Eighteen Holstein male calves that were fed a common diet were used. Calves were killed and ruminal epithelial tissue was collected and mounted in Ussing chambers under short-circuit conditions. For the measurement of the serosal-to-mucosal flux of urea ($J_{sm\text{-}urea}$) or mannitol ($J_{sm\text{-}mannitol}$), 26.25 kBq of ^{14}C-labelled urea and 37 kBq of ^{3}H-labelled mannitol were added to the serosal side resulting in final concentrations of 7 and 1 mmol/L, respectively. Steady-state isotope equilibration was assessed by taking flux measurements every 15 min for 2.5 h. The suitability of $NiCl_2$ and $HgCl_2$ as inhibitors of aquaporin function was assessed by the addition of 1 mmol/L $NiCl_2$ or $HgCl_2$ on the serosal and mucosal sides. Mucosal buffer pH was manipulated from 7.0 to 5.2 by the addition of gluconic acid. Steady-state $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ fluxes were observed by 45 min following isotopic additions to the serosal buffer. Ruminal epithelia collected from the caudal-dorsal sac had higher $J_{sm\text{-}urea}$ ($P = 0.03$) and lower $J_{sm\text{-}mannitol}$ ($P < 0.01$) than that collected from the ventral sac. Reducing mucosal buffer pH from 7.0 to 5.2 increased $J_{sm\text{-}urea}$ quadratically, where $J_{sm\text{-}urea}$ increased from pH 7.0 to 6.4 and thereafter decreased ($P = 0.01$). Changing mucosal buffer pH had no effect on $J_{sm\text{-}mannitol}$ ($P = 0.36$), tissue conductance (G_t; $P = 0.36$) or short-circuit current (I_{sc}; $P = 0.13$). Both $HgCl_2$ and $NiCl_2$ inhibited $J_{sm\text{-}urea}$ ($P < 0.01$); however, the addition of $HgCl_2$ increased G_t when compared to $NiCl_2$. When taken together, these results indicate that ruminal epithelia that are collected from the caudal-dorsal sac and are incubated under Ussing chamber

conditions with a mucosal pH of 6.4 and allowing 45 min for steady-state isotope equilibration is the most suitable experimental model for the study of urea transport. Also, when $NiCl_2$ is added to the serosal and mucosal sides at a concentration of 1 mmol/L, it is a potent inhibitor of aquaporin function without any negative effects on epithelial tissue integrity.

2.2 Introduction

In ruminants, urea that is recycled into the rumen is an important source of nitrogen (N) for microbial growth, particularly when dietary N supply is deficient (Lapierre and Lobley, 2001). Therefore, a better understanding of the physiological adaptation of urea transport is important to develop strategies that improve N efficiency in ruminants. For a long time, it was thought that urea passed from the bloodstream into the rumen by simple passive diffusion; however, it is now known that urea transporter (UT-B) proteins that are expressed in the ruminal epithelium play a role in urea transfer (Stewart *et al.* 2005; Abdoun *et al*. 2010; Doranalli *et al*., 2011). In these studies (Stewart *et al*. 2005; Abdoun *et al*. 2010; Doranalli *et al*. 2011), the functional role of UT-B in urea transfer across the ruminal epithelium was established by the serosal addition of phloretin (which is a known inhibitor of UT-B) to ruminal epithelial tissues that were mounted in Ussing chambers and then determining by difference the proportion of serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) that was phloretin-sensitive. These studies clearly demonstrated the inhibitory effects of phloretin on UT-B-mediated $J_{sm\text{-}urea}$. Aquaporins (AQP) are known to be predominantly involved in the movement of water across cell membranes (Ma and Verkman 1999); however, some AQP that are referred to as aquaglyceroporins (AQP-3, -7, -9, and -10) have also been demonstrated to be permeable to urea in non-ruminants (Bagnasco 2005). To determine their functional role in urea transfer in ruminants, I decided to use an in vitro approach that involved the use of inhibitors that would block AQP activity, similar to the use of phloretin to study UT-B function. Various studies have shown that divalent metal ions such as Hg^{2+} (Holm *et al*. 2004; Lakner *et al*. 2011) and Ni^{2+} (MacIver *et al*. 2009) are potent inhibitors of AQP function in various non-ruminant species. However, to my knowledge, the suitability or potency of these inhibitors in inhibiting AQP function in ruminal epithelial tissue incubated under Ussing chamber conditions has not been determined.

Ussing chamber studies of ruminal epithelial tissue have provided insight into the mechanisms that regulate serosal-to-mucosal urea transport; however, most studies (e.g., Abdoun

et al. 2010; Kiran *et al*. 2011; Lu *et al*. 2014) have used ovine ruminal epithelium and the optimum conditions that are required for the incubation of bovine ruminal epithelium in Ussing chamber, might be different from those employed in studies using ovine ruminal epithelia. An important consideration when conducting Ussing chamber experiments is the composition of the serosal and mucosal buffers that bath the ruminal epithelium (Clarke 2009). It is important that the buffers mimic, as much as possible, the composition and characteristics of blood and ruminal contents in order to obtain meaningful results. One of the characteristics of buffers that is important is pH. Serosal buffer pH is typically adjusted to 7.4, which mimics normal blood pH. On the other hand, work using mounted ruminal epithelial tissues obtained from lambs showed that maximal trans-epithelial flux of urea occurred at a mucosal pH near 6.2 (Abdoun *et al*. 2010). However, it has not been established if this mucosal pH will result in maximal trans-epithelial flux of urea when bovine ruminal epithelial tissue is used.

A second important consideration with the Ussing chamber approach appears to be the location of sampling of ruminal epithelial tissue. Regional differences in tissue morphology occur between compartments of the rumen (Hill *et al*. 2005); however, Waldron *et al. (*1991) demonstrated that SCFA metabolism was not different between cultured ruminal epithelial cells collected from either the caudal-dorsal or ventral sacs. In previous studies using the Ussing chamber technique, ruminal epithelial tissue were obtained from the ventral sac (Stewart *et al*., 2005; Abdoun *et al*., 2010; Doranalli *et al*., 2011) or caudal dorsal sac (Schurmann et al., 2014; Foote et al., 2014). Although the reasons were not discussed in these manuscripts, the choice of sampling location was probably based on the fact that the ventral sac is continuously exposed to ruminal fluid which could potentially influence its metabolic functions. However, visual observations from preliminary studies in my laboratory indicated that ruminal papillae density and size in cattle appeared to be highly variable from animal to animal when ruminal epithelial tissue was obtained from the ventral sac, whereas it was more uniform when ruminal epithelial tissue was obtained from the caudal-dorsal sac. Hill *et al. (*2005) reported similar morphological variations. When mounted in Ussing chambers, ruminal epithelial tissue that has large and non-uniform papillae tends to interfere with electrophysiology measurements as papillae may come in direct contact with electrode bridges and heterogeneous tissue induces more variability thereby changing statistical power. Therefore, it is important to determine whether site of collection impacts measurements made in vitro.

A third important consideration is the optimum time required for isotope equilibration with bovine ruminal epithelial tissue mounted in Ussing chambers. Flux studies with tissue preparations mounted in Ussing chambers measure the steady-state rate of transfer of metabolites of interest across the tissue, so an isotope equilibration period is necessary to reach that steady-state flux (Sehested *et al*. 1996; Clark 2008). If flux studies are performed before the isotope equilibration is achieved, then erroneous fluxes can be obtained (Sehested *et al*. 1996; Clark 2008). The length of the equilibration period appears to vary with the type of tissue preparation (Clark 2008) and likely substrate being transported. Previous studies using ovine ruminal epithelium collected from the ventral sac have allowed a 45-min isotope equilibration period for ^{14}C-urea (Abdoun *et al*. 2010); however, the length of the equilibration period that is needed for steady-state flux in bovine ruminal epithelium collected from the caudal-dorsal sac is unknown.

Therefore, the objectives of these experiments were 4-fold: 1) to determine if there are functional differences in urea flux and electrophysiology measurements between tissues obtained from the ventral sac and the caudal-dorsal sac of the rumen; 2) to determine the optimum mucosal pH for maximal trans-epithelial flux of urea for bovine ruminal epithelial tissue under Ussing chamber conditions; 3) to determine the optimum time required for isotope equilibration when using ^{14}C-urea and ^{3}H-mannitol as tracers in flux studies using bovine ruminal epithelial tissue under Ussing chamber conditions; and 4) to determine the suitability and potency of $HgCl_2$ and $NiCl_2$ as inhibitors of AQP function in ruminal epithelial tissue mounted under Ussing chamber conditions.

2.3 Materials and methods

2.3.1 Ethical approval

Experimental animals were cared for and handled in accordance with the guidelines in the Canadian Council of Animal Care (2009), and their use in this experiment was approved by the University of Saskatchewan Animal Research and Ethics Board (UCACS Protocol No. 20040048).

2.3.2 General procedures for flux measurements in the Ussing chamber

Eighteen male Holstein steers (213 ± 23.0 kg body weight; approximately 5 to 7 months of age) were housed individually in floor pens in the Livestock Research Building at the University of Saskatchewan. Steers were obtained from the University of Saskatchewan

Greenbrae dairy herd and were fed a diet consisting of 38% alfalfa hay, 31% barley silage, 20% rolled barley, and 11% protein, mineral and vitamin supplement (DM basis; 15.5% CP). Steers were fed daily at 0700 h for ad libitum intake (targeting 5 to 10% refusal of feed) and had free access to water.

To obtain ruminal epithelial tissue for Ussing chamber studies, calves were killed (one per day) by captive bolt stunning followed by pithing and exsanguination. Within 2 to 3 min of exsanguination, the digestive tract was removed from the abdominal cavity and a 300-cm^2 piece of ruminal epithelium was taken from the ventral sac and the caudal-dorsal sac, washed in a physiological buffer solution and the underlying muscular layer was gently removed. The ruminal epithelium was then placed in a physiological buffer solution maintained at 38ºC and transported the laboratory (approximately 20 min). The physiological buffer solution contained (mmol/L) 1 $CaCl_2 \cdot 2H_2O$, 1 $MgCl_2 \cdot 6H_2O$, 2 $Na_2HPO_4 \cdot 2H_2O$, 1 $NaH_2PO_4 \cdot H_2O$, 1 phenyl-phosphorodiamidate (urease inhibitor; ABCR), 5 butyric acid, 60 NaCl, 5 KCl, 10 glucose, 25 Na-acetate$\cdot 3H_2O$, 15 Na-gluconate, 10 Na-propionate, and 25 $NaHCO_3$ (Abdoun *et al*. 2010). Buffer pH was adjusted to pH 7.4 using 1 mol/L NaOH or 1 mol/L gluconic acid and was gassed with 95% O_2:5% CO_2 for 30 min prior to and during tissue transport.

Upon arrival at the laboratory, ruminal epithelia were cut into strips and placed between two halves of an Ussing chamber with an exposed surface area of 1.43 cm^2 or 3.14 cm^2, depending on the experiment. Ruminal epithelia were bathed in 10 mL of incubation buffer solutions on both the serosal and mucosal sides. Buffer solutions were identical to the physiological buffer but also contained penicillin G sodium salt (60 mg/L), kanamycin sulphate (100 mg/L), and fluorocytosine (50 mg/L) to inhibit bacterial and fungal growth. Mucosal buffer solutions were adjusted to the indicated pH by using 1 mol/L gluconic acid. Serosal and mucosal buffer solutions were circulated using gas lift (95% O_2:5% CO_2) and were maintained at 38.5ºC using water-jacket reservoirs. A computer-controlled voltage-clamp system (VCC MC6; Physiologic Instruments or Dipl.-Ing. K. Mussler, Scientific Instruments, Aachen, Germany, depending on the experiment) was used to maintain the ruminal epithelia under short-circuit conditions. Trans-epithelial conductance (G_t) was measured every 6 seconds and averaged for each flux period according to Doranalli *et al.* (2011). Mounted tissues were allowed 20 min for

equilibration of electrophysiology measurements (i.e., G_t and I_{sc}) before initiation of the experiment.

2.3.3 Validation of the optimum time required for isotope equilibration when using ^{14}C-urea and ^{3}H-mannitol as tracers

Holstein male calves (*n* = 6; 8 MOA, 315 ± 19.3 kg) were killed (one per day) as described above. Ruminal epithelial tissue was obtained from the caudal-dorsal sac and mounted in Ussing chambers as described above. Mounted tissues were bathed in the serosal and mucosal incubation buffers that had a pH of 7.4 and 6.4, respectively. Mounted tissues were allowed 20 min for equilibration of electrophysiology measurements. After that, 26.25 kBq of ^{14}C-labelled urea (Perkin-Elmer) and 37 kBq of ^{3}H-labelled mannitol (Perkin-Elmer) were added to the serosal side to achieve a final concentration of 1 mmol/L with a concentration gradient from the serosal to mucosal side. After the addition of isotopes, flux measurements were conducted over 2.5 h with 15-min flux periods. "Cold" samples (500 µL) were collected every 15 min and an equivalent volume of buffer was replaced to maintain a constant volume of buffer solutions on both the serosal and mucosal sides. Isotope fluxes at each sampling interval were then calculated to determine the point at which isotope equilibration occurred.

2.3.4 Validation of the optimum mucosal buffer pH for maximal trans-epithelial flux of urea in bovine ruminal epithelium

Holstein male steers (*n* = 6; 8 MOA, 315 ± 19.3 kg) were killed (one per day) as described above. Ruminal epithelial tissue was collected from the caudal-dorsal sac and was incubated in Ussing chambers under short-circuit conditions as described above, with an exposed surface area of 3.14 cm^2. Tissues were allowed to equilibrate for 20 min when bathed in the standard serosal and mucosal incubation buffers that had a pH of 7.4 and 6.4, respectively. After electrophysiology stabilization, mounted tissues (n = 4) were randomly assigned to 1 of 4 treatments which consisted of a mucosal buffer pH of 5.2, 5.8, 6.4 or 7.0. Tissues were allowed 5 min of equilibration after mucosal buffer substitution before the serosal addition of 26.25 kBq of ^{14}C-labelled urea (Perkin-Elmer) and 37 kBq of ^{3}H-labelled mannitol (Perkin-Elmer). Isotopes were allowed to equilibrate for 45 min before the start of the first flux period. Three consecutive 30-min flux periods were conducted. "Hot" samples (100 µL) were taken at the beginning and end of each flux period, while "cold" samples (500 µL) were taken every 30 min in order to

calculate $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, with replacement of an equivalent volume of buffer in order to maintain a constant volume of buffer solutions on both the serosal and mucosal sides.

2.3.5 Validation of the suitability and potency of $HgCl_2$ and $NiCl_2$ as AQP inhibitors in bovine ruminal epithelium

Holstein male calves (n = 3; 8 MOA, 293 ± 17.2 kg) were killed (one per day) as described above. Ruminal epithelial tissue was obtained from the caudal-dorsal sac and mounted in Ussing chambers with an exposed surface area of 1.43 cm^2. The composition of serosal and mucosal incubation buffers was as described above, using a mucosal pH of 6.4 and a serosal pH of 7.4. Mounted tissues were allowed 20 min for equilibration of electrophysiology measurements. After electrophysiology equilibration, individual ruminal epithelia (n = 1) were randomly assigned to 1 of 4 in vitro treatments i.e., control or bilateral addition of 0.5 mmol/L $HgCl_2$, 1.0 mmol/L $HgCl_2$, or 1.5 mmol/L $HgCl_2$, such that treatments were balanced for G_t (Doranalli *et al*., 2011). In order to measure the serosal–to–mucosal flux of urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$), 26 kBq of ^{14}C-labelled urea (Perkin-Elmer) and 37 kBq of ^{3}H-labelled mannitol (Perkin-Elmer) were added to the serosal buffer solutions resulting in final concentrations of 1 mmol/L of each urea and mannitol. 'Cold' samples (500 μL) were collected after a 45-min isotope equilibration period and at the beginning and end of each of three 30-min flux periods, buffer was replaced after sample collection. Samples from the 'hot' (serosal) side were collected before the first and after the last flux period, and buffer was not replaced. The first flux period was a baseline period, during which no treatment was imposed. At the beginning of the second flux period, $HgCl_2$ was dissolved in water and added to the serosal and mucosal buffers of 2 tissues that were randomly assigned to each $HgCl_2$ concentration. All chemicals (reagent grade), phloretin and antibiotics were obtained from Sigma-Aldrich unless otherwise stated.

For experiments with $HgCl_2$ and $NiCl_2$ three Holstein male calves (n = 3; 8 MOA, 293 ± 17.2 kg) were used. In these experiments, individual ruminal epithelia were mounted in Ussing chambers with an exposed surface area of 1.43 cm^2 and provided 20 min for stabilization of electrophysiology. Tissues were then randomly assigned to 1 of 3 in vitro treatments i.e., control or bilateral addition of 1 mmol/L $NiCl_2$, or 1 mmol/L $HgCl_2$ (n = 2) such that the treatments were balanced for mean G_t values (Doranalli *et al*., 2011). In order to measure the flux of urea and

mannitol ($J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, respectively), 26 kBq of ^{14}C-labelled urea (Perkin-Elmer) and 37 kBq of ^{3}H-labelled mannitol (Perkin-Elmer) were added to the serosal buffer solutions resulting in final concentrations of 1 mmol/L for urea and mannitol. 'Cold' samples (500 μL) were taken after a 45-min isotope equilibration period and at the beginning and end of each of three 30-min flux periods, and was with replacement of an equivalent volume of buffer in order to maintain a constant volume of buffer solutions on both the serosal and mucosal sides. Samples from the 'hot' (serosal) side were collected before the first and after the last flux period, this buffer was not replaced. The first flux period was a baseline period, during which no treatment was imposed. At the beginning of the second flux period, $HgCl_2$ or $NiCl_2$ (1 mmol/L) dissolved in water were added to the serosal and mucosal buffers of 2 tissues each.

In a third experiment, individual epithelia from 3 Holstein male calves (n = 3; 8 MOA, 293 ± 17.2 kg) were assigned to 1 of 3 treatments i.e., control or bilateral addition of 1 mmol/L $NiCl_2$, or 1 mmol/L $NiCl_2$ + 1 mmol/L phloretin in order to determine if $NiCl_2$ and phloretin inhibit separate pathways of urea transport. The $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were measured as described previously. The first flux period was used as a baseline period, and in vitro treatments were imposed during the second flux period. Inhibitors were dissolved in either water ($NiCl_2$) or 95% ethanol (phloretin), prior to being administered to the incubation buffers. Inhibitors were added to the serosal and mucosal buffers (n = 2 per animal) and were allowed to equilibrate for 5 min before continuation of the experiment.

2.3.6 Comparison of isotopic fluxes in ruminal epithelial tissues obtained from the ventral sac and caudal-dorsal sac

Holstein male calves (n = 6) were killed (one per day) as described above. Ruminal epithelial tissue from the ventral sac and the caudal-dorsal sac was collected and mounted in Ussing chambers with an exposed surface area of 3.14 cm^2. Ruminal epithelial tissues were incubated using the same buffer solution as described above, using a mucosal pH of 6.4 and a serosal pH of 7.4. Tissues were maintained under short-circuit conditions using a computer-controlled voltage-clamp (VCC MC6; Physiologic Instruments or Dipl.-Ing. K. Mussler, Scientific Instruments, Aachen, Germany). Tissue electrophysiology was allowed to equilibrate for 20 min before the addition of isotopic tracers. At the end of the equilibration period, 26.25 kBq of ^{14}C-labelled urea (Perkin-Elmer) and 37 kBq of ^{3}H-labelled mannitol (Perkin-Elmer)

were added to the serosal side resulting in final concentrations of 7 and 1 mmol/L, respectively. Thus, there was a serosal-to-mucosal urea concentration gradient to mimic the physiological conditions in the ruminant, where urea is not normally found in ruminal fluid (Doranalli *et al*., 2011). Isotope was allowed to equilibrate for 45 min before the start of the first flux period. Two consecutive 30-min flux periods were then conducted. "Cold" samples of 500 µL (mucosal side) were taken at the beginning and end of each flux period, while "hot" samples (serosal side) of 100 µL were taken at the beginning of flux period 1 and at the end of flux period 2. An equal volume of buffer solution was replaced for the cold samples to maintain a constant volume of buffer solutions on both the serosal and mucosal sides.

2.3.7 Calculations and statistical analysis

The $J_{sm\text{-}urea}$ and $J_{sm\text{-}manitol}$ fluxes were calculated according to Cotton and Reuss (1996). All flux and electrophysiology data were analyzed using the mixed procedure of SAS (SAS Institute, 2004). For determination of the effect of mucosal buffer pH on $J_{sm\text{-}urea}$, mucosal pH level was considered as a fixed effect while tissue replicate within calf was considered a random effect. Orthogonal polynomial contrasts were used to determine the effect of mucosal pH on flux and electrophysiology measurements. Since a quadratic equation was the most significant, the derivative of the polynomial equation was calculated, and then solved for the vertex in order to determine the calculated pH where maximal $J_{sm\text{-}urea}$ should be observed. In all cases presented above, significance was declared when $P \leq 0.05$ and trends were considered when $0.05 < P < 0.10$. In order to determine the effects of inhibitor on $J_{sm\text{-}urea}$, flux period and treatment were considered as fixed effects and tissue replicate within steer as a random effect. For the inhibitor studies, single degree of freedom contrasts were used to determine the effects of control vs. $HgCl_2$, and $HgCl_2$ vs. $NiCl_2$, and in the second experiment, control vs. $NiCl_2$, and $NiCl_2$ vs. $NiCl_2$ + phloretin. Electrophysiology and flux data from tissue location experiments were analyzed as a factorial design where individual tissue and inhibitor treatment were fixed effects and tissue replicate within calf was a random effect. Regression analysis was performed between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ for tissue location measures.

2.4 Results

2.4.1 Isotope Equilibration

Equilibration of ^{14}C- urea occurred at approximately 30 min, while equilibration of ^{3}H-mannitol occurred at 45 min (Figure 2.1).

2.4.2 Effect of varying mucosal pH

The $J_{sm\text{-}urea}$ increased from a mucosal pH of 5.2 to 6.4 (Quadratic, $P = 0.01$; Table 2.1), but decreased thereafter. The $J_{sm\text{-}mannitol}$ was not affected by mucosal pH ($P = 0.36$). Additionally, there was no effect of mucosal pH on G_t or I_{sc} ($P \geq 0.13$). The calculated vertex of the quadratic equation derivative depicted a maximal $J_{sm\text{-}urea}$ at a mucosal pH of 6.63.

2.4.3 Inhibitor Validation

The addition of either 1 mmol/L or 1.5 mmol/L $HgCl_2$ reduced $J_{sm\text{-}urea}$ ($P < 0.01$), whereas the addition of 0.5 mmol/L $HgCl_2$ was unable to reduce $J_{sm\text{-}urea}$ when compared to control chambers (Table 2.2). Tissue conductance was increased with all $HgCl_2$ additions ($P < 0.01$), whereas I_{sc} was only reduced in chambers containing 1.5 mmol/L $HgCl_2$ ($P < 0.01$). Both 1 mmol/L $HgCl_2$ and 1 mmol/L $NiCl_2$ had inhibitory effects on $J_{sm\text{-}urea}$ when added to the serosal and mucosal buffers in the Ussing chamber ($P < 0.01$; Table 2.3). Orthogonal contrasts showed that there was no difference in the inhibitable portions of $J_{sm\text{-}urea}$ when using 1 mmol/L $HgCl_2$ or 1 mmol/L $NiCl_2$ as inhibitors of AQP function ($P = 0.12$); however, the addition of 1 mmol/L $HgCl_2$ increased ($P < 0.01$) G_t compared to the addition of $NiCl_2$ (Table 2.3). The addition of $NiCl_2$ to the mucosal and serosal buffers tended ($P = 0.09$) to decrease $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, whereas the addition of $NiCl_2$ and phloretin together tended ($P = 0.07$) to depress $J_{sm\text{-}urea}$ even further (Table 2.4). There was no difference between $NiCl_2$ and $NiCl_2$ + phloretin for $J_{sm\text{-}mannitol}$ ($P = 0.52$); however, when the inhibitors were added together they tended ($P = 0.09$) to increase G_t, but I_{sc} was unaffected ($P = 0.14$).

2.4.4 Location of ruminal epithelial tissue sampling

The $J_{sm\text{-}urea}$ was greater in ruminal epithelia obtained from the caudal-dorsal sac than that obtained from the ventral sac ($P = 0.03$); however, $J_{sm\text{-}mannitol}$ was lower in ruminal epithelia that was obtained from the dorsal sac than that obtained from the ventral sac ($P < 0.01$; Table 2.5). Tissue conductance was not affected by the location of tissue sampling ($P = 0.65$), whereas I_{sc} was greater ($P = 0.05$) in ruminal epithelia obtained from the caudal-dorsal sac when compared

to that obtained from the ventral sac. There was no correlation between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ for ruminal epithelia obtained from both the caudal-dorsal sac ($r = 0.33$, $P = 0.29$) and ventral sac ($r = 0.39$, $P = 0.22$; data not shown). During this experiment, visual observations indicated that papillae size and variability was much greater in ventral sac, and that tissue collected from caudal-dorsal sac was much more uniform.

2.5 Discussion

The Ussing chamber is a versatile and proven research tool that has been applied in numerous studies to help elucidate mechanisms involved in transepithelial transport systems in many species (Clark 2009). In ruminants, the Ussing chamber approach has been used recently to study the factors that regulate the passage of urea across the ruminal epithelia (Stewart *et al*. 2005; Abdoun *et al*. 2010; Muscher *et al*. 2010; Doranalli *et al*. 2011). When using isotopic tracers to study transepithelial transport pathways in the isolated ruminal epithelia, it is important to allow a sufficiently long equilibration period for the isotope to reach a steady-state flux across the tissue (Clark 2009). In the current study with bovine ruminal epithelia, the results showed that isotope equilibration for ^{14}C-urea and ^{3}H-mannitol reached steady-state flux after 45 min. Other studies (Abdoun *et al*. 2010; Doranalli *et al*. 2011) in which $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ fluxes were measured with ^{14}C-urea and ^{3}H-mannitol as isotopic tracers, respectively, used a 45-min isotope equilibration period, thus confirming the present findings. The importance of reaching steady state flux across the epithelium under Ussing chamber experiments was outlined by Cotton and Reuss (1996), who indicated that unless isotope is crossing the epithelium at a constant and steady rate, calculating flux rates from the appearance of isotope on the 'cold' side would produce erroneous results. Studies with other isotopic tracers like ^{22}Na (Sehested *et al*. 1996) also reported that 45 min of equilibration was necessary before accurate flux measurement of ^{22}Na could be obtained.

Another major objective of this study was to determine the optimum mucosal pH for maximal $J_{sm\text{-}urea}$ when bovine ruminal epithelia are mounted in Ussing chambers. Abdoun *et al.* (2010) observed that, under short-circuit conditions, ovine ruminal epithelia collected from the ventral sac had a maximal $J_{sm\text{-}urea}$ when mucosal pH was 6.2. My results showed that $J_{sm\text{-}urea}$ doubled when mucosal pH was increased from 5.2 to 6.4, but decreased when mucosal pH was further increased to 7.0; thus, these results suggest that, based on the pH range in the present

study, the optimum mucosal pH for maximal $J_{sm\text{-}urea}$ was 6.4 when bovine ruminal epithelia obtained from the caudal-dorsal sac was incubated in Ussing chambers. Although only 4 data points were evaluated, the "bell-shaped" response that I observed in $J_{sm\text{-}urea}$ supports the previous observations of Abdoun *et al.* (2010) with ovine ruminal epithelia. Although the calculated maximal Jsm-urea came was 6.63, From a physiological standpoint, the stimulation of $J_{sm\text{-}urea}$ when mucosal pH is close to 6.4, which is indicative of active fermentation in the rumen, would ensure that greater rates of urea transfer coincide with greater availabilities of energy and C sources used for microbial amino acid synthesis and, hence, a transient increase in the demand for N to support microbial growth (Abdoun *et al*. 2007; 2010). On the other hand, the inhibition of $J_{sm\text{-}urea}$ when mucosal pH is <6.4 would ensure that blood urea is not transferred to the rumen where a depressed microbial population (due to the prevailing acidotic conditions) would have little use of the recycled N. This is in agreement with in vivo experiments by Simmons *et al.* (2009) that reported that tissue collected from steers fed a silage-based diet (ruminal pH = 6.99) had decreased expression of ruminal UT-B than steers fed concentrate-based diets (ruminal pH = 6.15). Simmons *et al.* (2009) further suggested that urea secretion into the rumen would be stimulated on the concentrate-based diet; however, they did not evaluated pH values specifically in their study. The results of the present study along with those of Abdoun *et al.* (2010) suggest that both low pH and high pH may inhibit transepithelial urea flux and it is likely that epithelial sensing mechanisms such as free fatty acid receptors may regulate this response (Lu *et al.*, 2014)

In the past, the functional roles of AQP in non-ruminant tissues have been investigated using $HgCl_2$ as an inhibitor (Holm *et al*. 2004; Liakopoulos *et al*. 2006; Yang *et al*. 2006; Ismail *et al*. 2009; Maclver *et al*. 2009); however, $HgCl_2$ has not been validated for use as a AQP inhibitor in bovine ruminal epithelia. Zelenina *et al.* (2004) reported that AQP are sensitive to most divalent metals, which induce a structural change in AQP thus inhibiting AQP-mediated transport. In *Xenopus laevis* oocytes overexpressing AQP1, AQP3 or AQP10 from European eels, Maclver *et al*. (2009) observed that 1 mmol/L of $HgCl_2$ was able to inhibit swelling when oocytes were immersed in a hypertonic solution by preventing AQP-mediated water movement into the cytosol. Holm et al. (2004) observed that the addition of $HgCl_2$ reduced glycerol and urea uptake by oocytes overexpressing AQP6. These observations are in agreement with the findings from the current study where the addition of 1 mmol/L $HgCl_2$ inhibited $J_{sm\text{-}urea}$. Because $HgCl_2$ is hazardous to work with and also poses challenges with proper waste disposal, in the

present study, I wanted to investigate the potency of safer divalent metals as inhibitors of AQP function. Zelenina *et al.* (2004) reported that the addition of $NiCl_2$ reduced the AQP-mediated urea flux in non-ruminant tissues. Based on this finding, the present study investigated the potency of $NiCl_2$ as an inhibitor of AQP-mediated urea transport in bovine ruminal tissue. The results demonstrated that $NiCl_2$ was equally potent in the inhibition of AQP-mediated $J_{sm\text{-}urea}$ when compared to $HgCl_2$ at an equivalent concentration. However, the addition of $HgCl_2$ increased G_t, while $NiCl_2$ had no effect on G_t, thus suggesting that $NiCl_2$ did not negatively affect tissue integrity. These observations are in agreement with previous work by Maclver *et al.* (2009), who reported that the use of $NiCl_2$ prevented the undesirable effects on membrane integrity that were observed with $HgCl_2$. To further validate the inhibitory effect of $NiCl_2$ on AQP-mediated $J_{sm\text{-}urea}$, the effect of $NiCl_2$ was combined to that of phloretin, a known inhibitor of UT proteins (Abdoun *et al*. 2010; Doranalli *et al*. 2011). When $NiCl_2$ and phloretin were added together, my results showed that their inhibitory effects on $J_{sm\text{-}urea}$ were additive, thus strongly suggesting that the phloretin- and $NiCl_2$-sensititive movement of urea across bovine ruminal epithelia occurred via distinctly-separate pathways. Because $NiCl_2$ presents fewer challenges with regards to safe handling as well as waste disposal when compared to $HgCl_2$, it is recommended that $NiCl_2$ be used in future experiments investigating the role of AQP in urea movement.

In previous studies (Stewart *et al*. 2005; Abdoun *et al*. 2010; Muscher *et al*. 2010; Doranalli *et al*. 2011) using sheep and goats, ruminal epithelial tissue was collected from the ventral sac; however, it is not clear if there are regional differences in $J_{sm\text{-}urea}$ across epithelial tissues collected from different locations within the bovine rumen. In the current study, $J_{sm\text{-}urea}$ was greater and $J_{sm\text{-}mannitol}$ was lower for epithelial tissue obtained from the caudal-dorsal sac compared to that obtained from the ventral sac, thus suggesting that there might be regional differences in the passage of urea from the bloodstream into the rumen. The reasons for the regional differences in $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ are unclear, but could be related to regional variations in metabolism and tissue morphology. It is well-established that the rumen is composed of ecological compartments which vary in terms of microbial populations, exposure to solid, liquid, and gaseous environments, and the concentrations of end-products of microbial fermentation (Czerkawski and Cheng 1988). Concentrations of SCFA and NH_3-N have been reported to be higher in liquid-containing compartments like the ventral sac when compared to

particulate-containing compartments like the caudal-dorsal sac (Czerkawski and Cheng 1988). Previous studies (Engelhardt *et al*. 1978; Rémond *et al*. 1993; Abdoun *et al*. 2009) have reported that increasing ruminal NH_3 concentration has inhibitory effects on urea transfer from the bloodstream into the rumen. Also, the presence of SCFA has been reported to have stimulatory effects on urea transfer into the rumen (Rémond *et al*. 1993; Abdoun *et al*. 2010; Lu *et al.* 2014). Regional differences in concentrations of SCFA and NH_3-N were not determined in the present study; however, Lu *et al.* (2015) reported that ruminal UT-B expression is transcriptionally up-regulated by SCFA and low pH. In turn, Zebeli *et al*. (2008) has shown that ruminal pH is lower and SCFA concentration is higher in the dorsal parts of the rumen. Linking these two findings together provides a plausible explanation for a higher expression of urea transporting proteins in the dorsal regions of bovine rumen. If the assumption is made that ruminal concentrations of SCFA and NH_3-N were higher in the caudal-dorsal sac when compared to the ventral sac as has been reported by others (Czerkawski and Breckenridge 1982), then the data would suggest that the stimulatory effects of SCFA and low pH on urea transfer could be partially attributed to the upregulation of UT-B expression in the caudal-dorsal sac compared to the ventral sac.

The regional differences in urea transfer that was observed in the present study could also be attributed to a different pattern of ruminal fluid exposure. Ruminal epithelia that is located in the ventral sac is continuously exposed to ruminal fluid, whereas ruminal epithelia that is located in the caudal-dorsal sac is intermittently exposed to ruminal fluid when animals lie down (~12 h per d; Gomez and Cook 2010) and during ruminal contractions (Waldron *et al*. 2002). As such, it may be speculated that intermittent exposure to low pH and high SCFA concentrations provides a stimulus for UT-B and/or AQP expression in the caudal-dorsal sac. Low ruminal pH has been linked with increased rumenitis (Nagaraja and Titgemeyer 2007) or erosion of the ruminal epithelium leading to a decrease in the barrier function of the tissue (Steele *et al*. 2012). Based on these observations, it is plausible that ruminal epithelia from the ventral sac exhibited reduced barrier function. Support for this assertion is provided by two observations from the present study: 1) that a greater $J_{sm\text{-}mannitol}$ was observed in ruminal epithelia obtained from the ventral sac when compared to that obtained from the caudal-dorsal sac; and 2) that G_t, which is a measure of tissue integrity (Clark 2009), changed in parallel with $J_{sm\text{-}mannitol}$. Mannitol is one of a group of hydrophilic solutes that are routinely used as markers of para-cellular transport or barrier function in Ussing chamber studies (Clark 2009). Penner *et al. (*2010) demonstrated that barrier

function (assessed using $J_{sm\text{-}mannitol}$) was reduced in ruminal epithelial tissue that was exposed to an acidotic challenge (mucosal pH of 5.2 for 60 min), thus supporting the present observations and the assumption that epithelia from the ventral sac may have been exposed to more acidic ruminal pH conditions. To my knowledge, only one published study (Waldron *et al*. 2002) has reported the effects of location of tissue sampling on vitro metabolism of ruminal epithelial cells. In that study, cells isolated from the anterior cranial pillar, ventral sac floor, caudal pillar surface, and caudal-dorsal sac ceiling were exposed to incubation solutions containing, among other substrates, propionate and butyrate. The origin of ruminal tissue had no effect on metabolite (β-hydroxybutyrate, acetoacetate, lactate, and pyruvate) production, suggesting that in the case of SCFA, the location of ruminal epithelia was not a key determinant of cell metabolism (Waldron *et al*. 2002). With transepithelial urea transfer, however, the present observations indicate that the origin of the ruminal epithelial tissue might need to be taken into consideration.

Ruminal epithelial tissue collected from the caudal-dorsal sac exhibited higher I_{sc} compared to that collected from the ventral sac, suggesting that that there were higher rates of electrogenic (active) transport in the caudal-dorsal sac. In vivo, SCFA in the non-dissociated state are able to cross the epithelial membranes by passive diffusion during periods of low ruminal pH; however, low ruminal pH also stimulates absorption of ruminal SCFA in the dissociated form via the SCFA/bicarbonate exchange pathway (Aschenbach *et al*. 2009). The influx of SCFA across the apical membrane either via passive diffusion or via SCFA/bicarbonate decreases the intracellular pH and stimulates apical Na^+/H^+ exchange. The latter leads via increased function of Na^+, K^+-ATPase to an increase in transepithelial Na^+ absorption, thus causing an increase in I_{sc} (Gäbel *et al.* 1991; Uppal *et al.* 2003). It is well established that dietary factors (low forage:concentrate ratio) can increased Na^+ absorption and an increase the stimulatory effect of ruminal SCFA on Na^+ absorption (Uppal *et al.* 2003). As such, the regional difference in I_{sc} could be due to a different stimulation of Na^+ absorption by the SCFA contained in the buffered incubation solution. Alternatively, it has been demonstrated that the majority of I_{sc} in the ruminal epithelium of sheep is carried by Na^+-dependent glucose transport when 10 mmol/L glucose is present in the mucosal incubation solution (Aschenbach et al. 2000).

Observations from these studies showed that: 1) the caudal-dorsal sac is a suitable tissue location for the collection of ruminal epithelia for incubation under Ussing chamber conditions;

2) a mucosal pH of 6.4 with a serosal pH of 7.4 results in maximal $J_{sm\text{-}urea}$ across the ruminal epithelia; 3) that a 45-min isotope equilibration period is sufficient to reach steady state flux across the epithelium; and 4) that $NiCl_2$ is a suitable for the inhibition of AQP mediated $J_{sm\text{-}urea}$ in the ruminal epithelium. Therefore, for the Ussing chamber experiments that were conducted for this thesis research, it was decided: 1) to use ruminal epithelial tissue that was collected from the caudal-dorsal sac; 2) for incubation buffers, use a serosal pH of 7.4 (to mimic blood pH) and a mucosal pH of 6.4; 3) utilized a 45-min incubation period; and 4) the use of a 1 $mmol^{-1} \cdot L$ $NiCl_2$ as an inhibitor of AQP in the ruminal epithelium.

Table 2.1. Effects of varying mucosal buffer pH on urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) fluxes, and tissue conductance (G_t) and short-circuit current (I_{sc}) in ruminal epithelium collected from the caudal-dorsal ruminal sac of Holstein calves (n = 6)

	Mucosal pH					Contrast *P* value	
Item	5.2	5.8	6.4	7.0	SEM	Linear	Quadratic
$J_{sm\text{-}urea}$ (nmol•cm^{-2}•h^{-1})	73.5	100.5	144.8	126.0	8.35	<0.01	0.01
$J_{sm\text{-}mannitol}$ (nmol•cm^{-2}•h^{-1})	90.9	85.4	75.6	81.5	14.2	0.36	0.53
G_t, mS·cm^{-2}	4.41	4.02	3.86	3.80	0.46	0.36	0.73
I_{sc}, mA·cm^{-2}	11.5	8.1	15.3	13.4	2.2	0.13	0.71

Concentrations of urea and mannitol were 7 and 1 mmol/L, respectively.

Table 2.2. Effects of the varying mucosal and serosal concentrations of $HgCl_2$on urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) fluxes, and tissue conductance (G_t) and short-circuit current (I_{sc}) in

	Concentration of $HgCl_2$ ($mmol \cdot L^{-1}$)					
	0.0	0.5	1.0	1.5	SEM	*P* value
$J_{sm\text{-}Urea}$, $nmol \cdot (cm^2)^{-1} \cdot h^{-1}$	85.2a	71.1a	49.1b	45.7b	10.8	<0.0001
Gt, mS	3.11b	6.82a	6.28a	6.43a	0.76	0.007
Isc, mA	18.2a	14.6ab	13.2ab	7.58b	2.37	0.005

ruminal epithelium collected from Holstein calves.

Table 2.3. Effects of the serosal and mucosal addition of $HgCl_2$ (1 mmol/L) and $NiCl_2$ (1 mmol/L) on urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) fluxes, and tissue conductance (G_t) and short-circuit current (I_{sc}) in ruminal epithelium collected from the caudal-dorsal ruminal sac of Holstein calves (n = 3)

	Treatment				Contrast *P* value	
Item	Control	$HgCl_2$	$NiCl_2$	SEM	Control vs. $HgCl_2$	$HgCl_2$ vs. $NiCl_2$
$J_{sm\text{-}Urea}$, $nmol\cdot(cm^2)^{-1}\cdot h^{-1}$	78.8	51.3	40.9	10.8	<0.01	0.12
G_t, $mS\cdot cm^{-2}$	4.81	6.87	5.16	0.93	<0.01	<0.01
I_{sc}, $mA\cdot cm^{-2}$	12.7	13.1	11.8	2.62	0.26	0.21

Concentrations of urea and mannitol were 7 and 1 mmol/L, respectively.

Table 2.4. Effects of the aquaporin and urea transporter inhibitor addition on urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) fluxes, and tissue conductance (G_t) and short-circuit current (I_{sc}) in ruminal epithelium collected from Holstein calves (n = 3).

Item	Control	$NiCl_2$	$NiCl_2$ + Phloretin	SE	*P* value	
					C vs. $NiCl_2$	$NiCl_2$ vs. $NiCl_2$ + Phloretin
$J_{sm\text{-}Urea}$, nmol·$(cm^2)^{-1}$·h^{-1}	87.41	56.58	23.08	11.51	0.09	0.072
$J_{sm\text{-}Mannitol}$, nmol·$(cm^2)^{-1}$·h^{-1}	43.01	23.92	18.52	6.08	0.07	0.52
Gt, mS	2.45	1.89	4.57	0.84	0.64	0.09
Isc, mA	15.1	14.1	9.26	2.68	0.87	0.14

Table 2.5. Effects of the location of ruminal tissue sampling on urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) fluxes, tissue conductance (G_t) and short-circuit current (I_{sc}) in Holstein male calves (n = 6)

	Tissue			
Item	Ventral sac	Caudal-dorsal sac	SE	*P* value
$J_{sm\text{-}urea}$ (nmol•cm^{-2}•h^{-1})	79.1	89.7	4.07	0.03
$J_{sm\text{-}mannitol}$ (nmol•cm^{-2}•h^{-1})	16.0	8.90	0.06	<0.01
G_t (mS·cm^{-2})	12.75	8.77	6.31	0.65
I_{sc} (mA·cm^{-2})	12.5	15.6	1.27	0.05

Concentrations of urea and mannitol were 7 and 1 mmol/L, respectively.

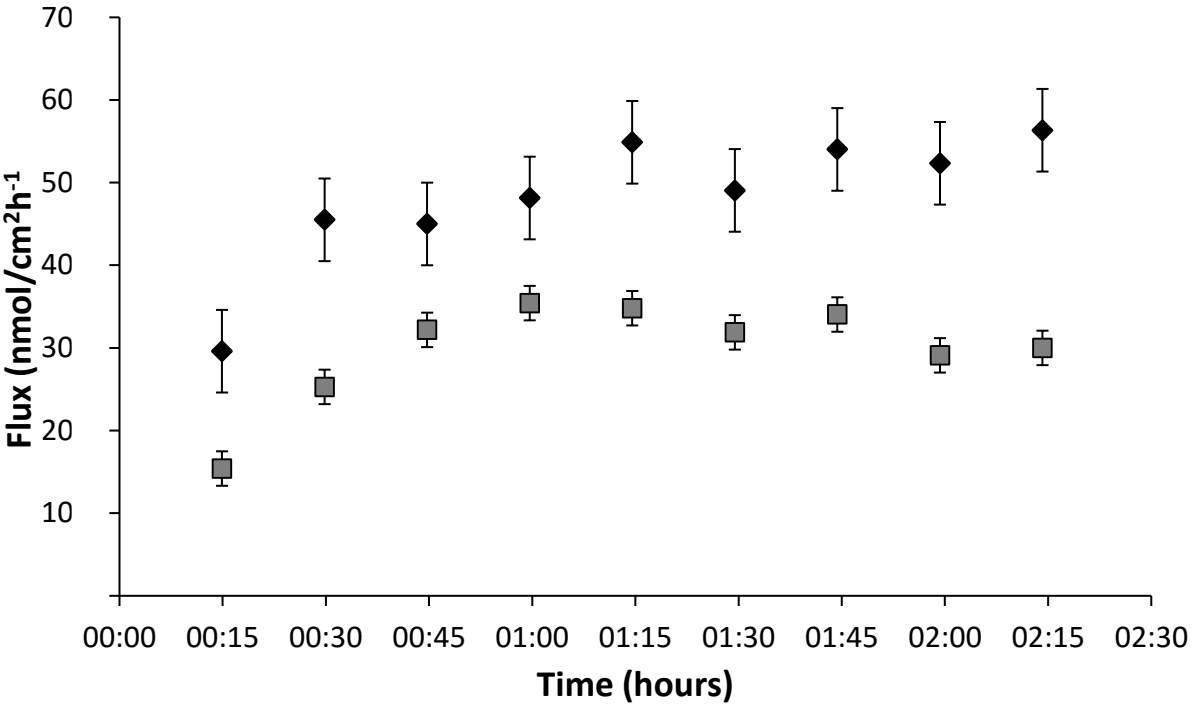

Figure 2.1. Flux measurements of ^{14}C-urea (diamonds, $J_{sm\text{-}urea}$) and ^{3}H-mannitol (squares, $J_{sm\text{-}mannitol}$) in ruminal epithelia from the caudal-dorsal ruminal sac of male Holstein calves (n = 3). Concentrations of urea and mannitol were 7 and 1 mmol/L, respectively. Error bars represent the variation in flux sampled at each time point.

CHAPTER 3. SEROSAL-TO-MUCOSAL UREA FLUX IS MEDIATED VIA PHLORETIN- AND $NiCl_2$-SENSITIVE PATHWAYS IN THE ISOLATED BOVINE RUMEN EPITHELIUM[1]

3.1 Abstract

Urea transport (UT-B) proteins are known to facilitate urea movement across the ruminal epithelium; however, other mechanisms may be involved as well because inhibiting UT-B does not completely eliminate urea transport. Of the aquaporins (AQP), which are a family of membrane-spanning proteins that are predominantly involved in the movement of water, AQP-3, -7, and -10 are also permeable to urea, but it is not clear if they contribute to urea transport across the ruminal epithelium. The objectives of this study were to determine: 1) the functional roles of AQP and UT-B in the serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) across rumen epithelium; and 2) whether functional adaptation occurs in response to increased diet fermentability. Twenty five Holstein steer calves (n = 5) were assigned to a control diet (CON; 91.5% hay and 8.5% vitamin and mineral supplement) or a medium grain diet (MGD; 41.5% barley grain, 50% hay, and 8.5% vitamin and mineral supplement) that was fed for 3, 7, 14, or 21 d. Calves were killed and ruminal epithelium was collected for mounting in Ussing chambers under short-circuit conditions and for analysis of mRNA abundance of UT-B and AQP-3, -7, and -10. To mimic physiologic conditions, the mucosal buffer (pH 6.2) contained no urea, while the serosal buffer (pH 7.4) contained 1 m*M* urea. The fluxes of ^{14}C-urea ($J_{sm\text{-}urea}$; 26 kBq/10 mL) and ^{3}H-mannitol ($J_{sm\text{-}mannitol}$; 37 kBq/10 mL) were measured, with $J_{sm\text{-}mannitol}$ being used as an indicator of paracellular or hydrophilic movement. Serosal addition of phloretin (1 mmol/L) was used to inhibit UT-B-mediated urea transport, while $NiCl_2$ (1 mmol/L) was used to inhibit AQP-mediated urea transport. Across treatments, the addition of phloretin or $NiCl_2$ reduced the $J_{sm\text{-}urea}$ from 116.5 to 54.0 and 89.5 nmol·cm^{-2}·h^{-1}, respectively. When both inhibitors were added simultaneously, $J_{sm\text{-}urea}$ was further reduced to 36.8 nmol·cm^{-2}·h^{-1}. Phloretin-sensitive and $NiCl_2$-sensitive $J_{sm\text{-}urea}$ were not affected by diet. The $J_{sm\text{-}urea}$ tended to increase linearly as the duration of adaptation to MGD increased, with the lowest $J_{sm\text{-}urea}$ being observed in animals fed CON (107.7 nmol·cm^{-2}·h^{-1}) and the highest for those fed the MGD for 21 d (144.2 nmol·cm^{-2}·h^{-1}). Phloretin-insensitive $J_{sm\text{-}urea}$ tended to increase linearly as the duration of adaptation to MGD

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increased, whereas there was a tendency for $NiCl_2$-insensitive $J_{sm\text{-}urea}$ to be affected by diet. Gene transcript abundance for AQP-3 and UT-B in ruminal epithelium increased linearly as the duration of MGD adaptation increased. For AQP-7 and AQP-10, gene transcript abundance in animals that were fed the MGD was greater when compared to CON animals. These results demonstrate that both AQP and UT-B play significant functional roles in urea transport, and they may play a role in the urea transport during dietary adaptation to fermentable carbohydrates.

3.2 Introduction

Ruminants are able to maintain a positive nitrogen (N) balance by recycling blood urea-N to the gastrointestinal tract (GIT), a physiological mechanism that is referred to as urea-nitrogen salvaging (UNS; Fuller and Reeds, 1998). Various studies (e.g., Lobley et al., 2000; Marini et al., 2004; Gozho et al., 2008; Kiran and Mutsvangwa, 2011) have demonstrated that substantial amounts of N transit the urea-N pool daily in ruminants, and reported data indicate that 40 to 80% of endogenously-produced urea-N is returned to the GIT (Hartmeyer and Martens, 1980; Lapierre and Lobley, 2001). Urea can enter all major GIT compartments directly across the GIT wall (Egan et al., 1986; Siddons et al., 1985), but the reticulo-rumen is the GIT compartiment where most of the use of recycled urea-N for microbial protein synthesis occurs (Lapierre and Lobley, 2001). Urea-N that passes into the reticulo-rumen provides N for microbial growth, thus contributing amino acids to the animal when ruminal microbes pass out of the rumen and are subsequently digested at the small intestine (Lobley et al., 2000).

Ritzhaupt et al. (1997; 1998) demonstrated the presence of carrier-mediated, facilitative urea transporter (UT) proteins in ovine ruminal and colonic epithelia which are now known to allow the rapid movement of urea down a concentration gradient from blood into the GIT. These UT are derived from 2 major gene variants, namely UT-A and UT-B (Stewart et al., 2005), and mRNA expression for UT-B has been characterized in ruminal epithelium (Marini and Van Amburgh, 2003; Marini et al., 2004; Stewart et al., 2005). Various researchers (Stewart et al, 2005; Abdoun et al., 2010; Kiran et al., 2011) showed that adding phloretin (an inhibitor of UT-B function) to mounted ruminal epithelia in Ussing chambers reduced the serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$), indicating that UT-B have a functional role in UNS; however, a variable portion of $J_{sm\text{-}urea}$ (up to 50%) was not phloretin-inhibitable, which suggests that other phloretin-insensitive mechanisms for urea transport exist in ruminal epithelia. Possible alternative transport

mechanisms are the aquaporins (AQP), which are a family of membrane-spanning proteins that are predominantly involved in the movement of water and are expressed in many tissues (including the GIT) of non-ruminants (Ma and Verkman, 1999). Some AQP that are referred to as aquaglyceroporins (AQP-3, -7, -9, and -10) have been shown to be permeable to urea in non-ruminants (Rojek et al., 2008). Røjen et al. (2011a) showed that AQP-3, -7, and -10 are expressed in bovine ruminal epithelium; however, surprisingly, the mRNA expression of these AQP was greater in cows fed a high N compared to those fed a low N diet. This was discordant with the increased trans-epithelial urea transfer that was observed when dietary N content was decreased in the same study (Kristensen et al., 2010), leading the authors to suggest that AQP had limited involvement in trans-epithelial urea transport. Although this study showed no relationship between the mRNA abundance or protein expression of the AQP-3, -7, and -8 and ruminal epithelial permeability to urea, there have been no studies to my knowledge that have examined the functional roles of these AQP in urea movement across the ruminal epithelium. Also, it is unknown if AQP and UT-B expression is regulated in an integrated manner that is designed to conserve N via UNS.

It is well-established that the entry of urea-N into the GIT increases when ruminants are fed diets high in ruminally-fermentable carbohydrate (Huntington and Archibeque, 1999) or when carbohydrate digestion is shifted from the small intestine to the rumen via grain processing (Delgado-Elorduy et al., 2002; Theurer et al., 2002). Under these feeding conditions, improved ammonia-N uptake by ruminal microbes decreases its portal absorption and, consequently, blood urea-N concentrations decrease. Because the movement of urea-N from blood across the ruminal wall into the lumen is positively correlated with blood urea-N concentration (Vercoe, 1969; Sunny et al., 2007), lower blood urea-N concentrations do not provide a favorable concentration gradient; thus, it is plausible that the increased passage of urea-N into the rumen when energy availability is increased could be mediated via changes in the expression of membrane proteins like UT-B and AQP, which would result in changes in ruminal epithelial permeability to urea-N (Abdoun et al., 2010). In support of this assertion, the addition of short-chain fatty acids at a luminal pH of 6.4 (which is indicative of high rates of ruminal carbohydrate fermentation) to ruminal epithelial tissue mounted in Ussing chambers resulted in a 4-fold increase in $J_{sm\text{-}urea}$. What is unknown, however, is if UT-B and AQP mediate the known effects of ruminal carbohydrate digestion on urea-N passage from blood into the rumen.

The aim of the current study was to determine the relative functional roles of UT-B and AQP for urea transport across the ruminal epithelium, and to determine if UT-B and AQP mediate the known effects of ruminal carbohydrate digestion on $J_{sm\text{-}urea}$ after an abrupt change in diet fermentability. My hypothesis was that UT-B and AQP play functional roles in UNS, and that the increase in $J_{sm\text{-}urea}$ that has been observed in ruminants fed more fermentable carbohydrates is mediated via changes in UT-B and AQP function.

3.3 Materials and Methods

3.3.1 Animals, experimental treatments and feeding management

The detailed descriptions of experimental procedures are outlined in Schurmann et al., (2014). Briefly, twenty five Holstein steer calves were used in this study. Steer calves (BW = 213 ± 23.0 kg) were housed in the Livestock Research Building at the University of Saskatchewan, and their use in this experiment was pre-approved by the University of Saskatchewan Animal Care Committee (Protocol No. 20100021). Before the beginning of the experiment, calves were fed a high forage diet (91.5% grass hay and 8.5% mineral and vitamin supplement). At the beginning of the experiment, calves were weighed and then blocked by body weight into 5 blocks of 5 animals per block. Within each block, calves were then assigned to 1 of 5 dietary treatments which consisted of a control diet (designated CON; 91.5% hay and 8.5% vitamin and mineral supplement; 10.6% CP, 4.1% Starch, 48.4% NDF) or a moderate grain diet (designated MGD; 41.5% barley grain, 50% hay, and 8.5% vitamin and mineral; 11.4% CP, 24.6% Starch, 33.5% NDF) that was fed for 3, 7, 14, or 21 d (designated G3, G7, G14, and G21, respectively). Because only one animal could be killed per day to obtain tissues for ex vivo studies, it was necessary that the initiation of the feeding of experimental diets to individual animals within each block was staggered over time to achieve the desired lengths of dietary exposure (i.e., 0, 3, 7, 14, or 21 d). Within each block, the order of killing was random. This experimental design ensured that the order of killing was balanced among treatments over time. Animals were fed at 2.25% of BW (DMI = 4.8 ± 0.2 kg/d), once daily at 0800 h and had free access to water throughout the duration of the experiment.

3.3.2 Flux measurements in Ussing chambers

After the desired length of dietary exposure, calves were killed (one per day) by captive bolt stunning followed by pithing and exsanguination at 2 h after feeding, allowing time for

fermentation to occur in the reticulo-rumen from the morning feeding (Robinson et al. 1997). Within 2-3 min of exsanguination, the digestive tract was removed from the abdominal cavity and a 300-cm^2 piece of ruminal wall was removed from the caudal-dorsal blind sac. This area of the ruminal epithelium was chosen due to its uniformity in papillae size and density. The ruminal wall was washed in a physiological buffer solution until clean. The physiological buffer solution (pH 7.4; 38ºC) was continuously gassed with carbogen (95% O_2/5% CO_2) and it contained (mmol/L): 1 $CaCl_2 \cdot 2H_2O$, 1.25 $MgCl_2 \cdot 6H_2O$, 15.6 NaCl, 5.5 KCl, 0.6 $NaH_2PO_4 \cdot H_2O$, 2.4 $Na2HPO_4 \cdot 2H_2O$, 10 acetic acid, 5 Na-D/L-lactate (60%), 1 L-glutamine, 10 HEPES-free acid, 10 Na-propionate, 10 Na-butyrate, 10 NaOH, and 24 $NaHCO_3$. After washing, the ruminal wall was gently stripped of the underlying muscular layer. The epithelium was then placed in the physiological buffer solution and transported to the laboratory (approximately 5 min). The physiological buffer solution was continuously gassed with carbogen during tissue transport. Upon arrival at the laboratory, ruminal epithelia were cut into strips and placed between two halves of an Ussing chamber with an exposed surface area of 1.43 cm^2. For each steer, six Ussing chambers were used for in vitro studies. Ruminal epithelia were bathed in 10 mL of isolated incubation buffer solutions on both the serosal and mucosal sides. The incubation buffer solution contained (m*M*): 1 $CaCl_2 \cdot 2H_2O$, 1 $MgCl_2 \cdot 6H_2O$, 2 $Na_2HPO_4 \cdot 2H_2O$, 1 $NaH_2PO_4 \cdot H_2O$, 1 phenyl-phosphorodiamidate (urease inhibitor; Alfa Aesar, Ward Hill, MA), 5 butyric acid, 60 NaCl, 5 KCl, 10 glucose, 25 Na-acetate$\cdot 3H_2O$, 15 Na-gluconate, 10 Na-propionate, and 25 $NaHCO_3$ (Abdoun et al., 2010). Antibiotics (penicillin G sodium salt, 60 $mg \cdot L^{-1}$; kanamycin sulphate, 100 $mg \cdot L^{-1}$; and flurocytosine, 50 $mg \cdot L^{-1}$) were also added to the incubation buffer solution to inhibit urease activity (Doranalli et al., 2011). Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO). Serosal and mucosal buffer solutions were adjusted to pH 7.4 and 6.4, respectively, using 1 m*M* NaOH or 1 m*M* HCl, to mimic in vivo physiological conditions. A mucosal pH of 6.4 was chosen because it is indicative of high rates of ruminal carbohydrate fermentation and preliminary studies with bovine ruminal epithelia showed that maximal $J_{sm\text{-}urea}$ was achieved at that pH (Chapter 2). Also, Abdoun et al. (2010) observed that $J_{sm\text{-}urea}$ in isolated ovine ruminal epithelia was maximal at that pH. Serosal and mucosal incubation buffer solutions were mixed using gas lift (95% O_2/5% CO_2) and were maintained at 38ºC using water-jacket reservoirs. A computer-controlled voltage-clamp system (VCC MC6; Physiologic Instruments) was used to maintain the ruminal epithelia under short-

circuit conditions. Trans-epithelial conductance (G_t) was measured every 6 seconds and mean Gt values were then calculated for each flux period (Doranalli et al. 2011).

For each calf, 20 min was allowed for the stabilization of electrophysiology before flux measurements were initiated. For the measurement of serosal-to-mucosal urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) fluxes, a solution that was spiked with ^{14}C-urea (26 kBq; Perkin Elmer, Waltham, MA) and ^{3}H-mannitol (37 kBq; Perkin Elmer) was then added to the serosal side to achieve final concentrations of 1 m*M* for both urea and mannitol. Mannitol was used to determine the magnitude of paracellular pathways (Stewart et al., 2005; Abdoun et al., 2010). Because urea was not added to the mucosal side, a serosal-to-mucosal concentration gradient existed. The rationale for this approach was 2-fold: 1) urea that is secreted into the rumen from the bloodstream is rapidly degraded by epimural ureolytic bacteria, such that ruminal fluid typically does not contain urea (Muscher et al., 2010); and 2) previous studies have demonstrated that a serosal-to-mucosal urea concentration gradient facilitates the movement of urea from the bloodstream into the rumen (Vercoe, 1969; Sunny et al., 2007). Our laboratory has used a similar approach previously (Kiran et al., 2011). A serosal urea concentration of 1 m*M* was used because previous studies (Abdoun et al., 2010; Kiran et al., 2011) have clearly shown that such a concentration can elicit transport changes under Ussing chamber conditions. After the addition of isotopic tracers, 30 min was allowed for isotope equilibration before the administration of in vitro treatments. Individual ruminal epithelia (N = 6, n = 2) were assigned to 1 of 3 ex vivo treatments, which were control (no inhibitor added), or the addition of phloretin or $NiCl_2$. Phloretin and $NiCl_2$ were dissolved in ethanol and were added to the serosal and mucosal buffers to achieve a final concentration of 1 m*M*, with an equivalent volume of ethanol being added to the serosal side for the control epithelia. Phloretin (Stewart et al., 2005; Abdoun et al., 2010) and $NiCl_2$ (Maclver et al., 2009) are inhibitors of UT and AQP function, respectively, and were used to determine inhibitor-sensitive $J_{sm\text{-}urea}$. After the addition of inhibitors, an additional 15 min was allowed for inhibitor equilibration before $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ across ruminal epithelia were measured during a 30-min flux period (flux period 1). Subsequently, a second 30-min flux period (flux period 2) was performed after 1 m*M* $NiCl_2$ was added to the chambers previously treated with 1 m*M* phloretin, and vice versa. This was done in order to determine the combined effects of phloretin and $NiCl_2$ on $J_{sm\text{-}urea}$.

3.3.3 Tissue collection and preservation for mRNA expression

Simultaneously to the collection of ruminal epithelial tissue for Ussing chamber experiments, ruminal epithelial tissue samples were also collected from the caudal-dorsal sac for gene expression measurements. Ruminal epithelial tissue was washed three times in ice-cold PBS solution, transferred into a container filled with RNAlater, temporarily stored at 4°C for 24 hours, before being frozen at -20ºC until analysis for UT-B, and AQP-3, -7, and -10 mRNA abundance. Briefly, tissue samples were homogenized and RNA was then extracted using Trizol (Invitrogen, Burlington, ON, Canada) as described by Chomczynski and Sacchi (1987). The RNA concentration was determined by measuring the absorbance at 260 and 280 nm using a NanoDrop (ND-1000, NanoDrop Technologies, Wilmington, DE). Quantitative real-time PCR (qRT-PCR) was conducted using a Mx3005 real-time PCR machine (Agilent, Mississauga, ON, Canada) with a 10-min pre-incubation at 95ºC followed by 40 cycles consisting of 15 sec at 95ºC and 60 sec at 60ºC. All samples were analyzed in duplicate. The primers used for UT-B, and AQP-3, -7, and -10 have been reported previously (Røjen et al., 2011a).The threshold cycle (CT) was deemed to be the point at which the florescent signal of the PCR product crossed the threshold. The average CT for each cow and gene was compared with each cow's respective average CT for the housekeeping gene (GAPDH) by subtracting CT of GAPDH from the CT of the target gene to calculate ΔCT. To compare the expression of genes between treatments, one calf from the control treatment was selected at random and was used as the calibrator. The ΔCT of this animal was used as a reference value to calculate ΔΔCT, which generated a ΔΔCT value of 0 for this calibrator animal. Fold-change of target gene expression was then calculated as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001; Ontsouka et al., 2004).

3.3.4 Ruminal fluid and blood collection, and analysis

Ruminal contents were collected at slaughter from the caudal-dorsal blind sac and strained through 2 layers of cheesecloth to obtain ruminal fluid. A 10-mL sample was preserved with 2 mL of 1% sulfuric acid for ruminal NH_3-N analysis. Samples were stored at -20ºC until analysis. Ruminal NH_3-N was determined using a phenol- hypochlorite assay (Broderick and Kang, 1980). Blood samples were collected by jugular venipuncture into 6-mL EDTA-coated vacutainer tubes (Becton Dickinson) 5 min prior to slaughter. Samples were placed on ice until being centrifuged at 3,000 x *g* for 15 min at 4ºC. Serum was separated and frozen at -20ºC until analysis. Serum was analyzed for urea-N using the diacetyl monoxime method (Marsh et al.,

1957) using a commercial kit (Stanbio Urea Nitrogen Kit, Procedure No. 0580; Stanbio Laboratories, Boerne, TX)

3.3.5 Calculations and Statistical Analysis

All flux, electrophysiology, and mRNA abundance data were first analyzed using the mixed procedure of SAS version 9.2 (SAS Institute, Cary NC) with block and treatment (CON, G3, G7, G14, G21) considered fixed effects, and calf as random. For this analysis, sub-treatment data were used to determine the pathway of $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ flux. The total $J_{sm\text{-}urea}$ and total $J_{sm\text{-}mannitol}$ were obtained from chambers that had no inhibitor added to incubation buffer (control chambers). The effect of flux period was evaluated to determine potential tissue changes with lengthening time under incubation conditions, period was deemed insignificant and removed from the model. The phloretin- and $NiCl_2$-insensitive $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were obtained from chambers with inhibitors added to the incubation buffer during flux period 1. The phloretin- and $NiCl_2$-sensitive $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were then calculated as the difference between total flux and phloretin-and $NiCl_2$-insensitive fluxes. Inhibitor-insensitive $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were obtained from chambers with both inhibitors present during flux period 2. The inhibitor-sensitive $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were then calculated as the difference between total flux and inhibitor-insensitive fluxes. Polynomial contrasts were used to determine whether the response was linear, quadratic, or cubic (CON to G21), and whether there was a difference between the CON and G21 treatments. The polynomial contrasts were constructed to account for unequal spacing for days on the MGD treatment.

Data for flux measurements were also analyzed using the same model described above except that sub-treatment was included in the model and the interaction between treatment and sub-treatment was assessed. The interaction between treatment and sub-treatment was not significant and was removed from the model. Data for $J_{sm\text{-}mannitol}$ did not differ by flux pathway and the data were not presented.

The relationships between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, $J_{sm\text{-}urea}$ and ruminal NH_3-N concentration, $J_{sm\text{-}urea}$ and plasma urea-N, and $J_{sm\text{-}urea}$ and Gt were tested using the correlation procedure of SAS (SAS Institute). Studentized residuals were used to determine and eliminate outliers from the dataset. For all analyses, significance was declared when $P < 0.05$, and trends were considered when $0.05 < P < 0.10$.

3.4 Results

3.4.1 Diet effects on ruminal NH_3-N and plasma urea-N concentrations

Nitrogen intake was unaffected by diet (Table 3.1). Increasing days on MGD altered ruminal NH_3-N concentrations in a cubic manner ($P = 0.004$); however, plasma urea-N concentrations were unaffected ($P = 0.19$) by dietary treatment (Table 3.1).

3.4.2 Inhibitor and diet effects on transepithelial flux and tissue electrophysiological measurements in the Ussing chambers

Across dietary treatments, total $J_{sm\text{-}urea}$ was 116.5 nmol·cm^{-2}·h^{-1} and the addition of phloretin or $NiCl_2$ reduced ($P < 0.001$) the $J_{sm\text{-}urea}$ by 54% and 23%, respectively (Figure 3.2). As well, phloretin-sensitive and $NiCl_2$-sensitive $J_{sm\text{-}urea}$ were different ($P < 0.001$) from each other. When both inhibitors were added together, total inhibitor-sensitive $J_{sm\text{-}urea}$ was lower when compared to $NiCl_2$-sensitive ($P < 0.001$) or phloretin-sensitive ($P = 0.028$) $J_{sm\text{-}urea}$, reducing $J_{sm\text{-}urea}$ by 68% when compared to total flux. The sequence of inhibitor addition had no effect on total-insensitive $J_{sm\text{-}Urea}$ ($P = 0.69$). The correlation relationships between simultaneously-measured $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ fluxes were significant for total insensitive ($r = 0.89$; $P < 0.001$; Figure 3.2), as well as phloretin-insensitive ($r = 0.91$; $P < 0.001$) and $NiCl_2$-insensitive ($r = 0.9$; $P < 0.001$) fluxes (data not shown).

The effects of length of dietary adaptation on $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ fluxes, and tissue electrophysiological measurements are presented in Table 3.2. The $J_{sm\text{-}urea}$ ($P = 0.096$) and $J_{sm\text{-}mannitol}$ ($P = 0.14$) were not different between calves that received the MGD for 21 d (G21) and those that were not fed grain (CON). However, $J_{sm\text{-}urea}$ ($P = 0.075$) and $J_{sm\text{-}mannitol}$ ($P = 0.058$) tended to increase linearly with increasing time on the MGD. Feeding grain tended to increase the $NiCl_2$-sensitive $J_{sm\text{-}urea}$ ($P = 0.07$); however, this was not observed for $NiCl_2$-insensitive $J_{sm\text{-}urea}$ ($P = 0.11$). Phloretin-insensitive $J_{sm\text{-}urea}$ tended to increase linearly with increasing days on the MGD ($P = 0.075$). Phloretin sensitive $J_{sm\text{-}urea}$ was not affected by increasing diet fermentability ($P = 0.54$). Diet had no effect on tissue conductance (G_t); however, there was a significant cubic effect ($P = 0.02$) on short-circuit current (I_{sc}).

3.4.3 Diet effects on UT-B and AQP mRNA abundance in ruminal epithelium

The mRNA expression of UT-B ($P = 0.007$) and AQP-3 ($P = 0.001$) in ruminal epithelium increased linearly as the duration of adaptation to the MGD increased (Table 3.3). For

AQP-7 (P = 0.008) and AQP-10 (P = 0.009), gene transcript abundance in animals that were fed the MGD diet for 21 d was greater when compared to CON animals. Total $J_{sm\text{-}urea}$ was significantly correlated with mRNA abundance of both AQP-3 (r = 0.72; P = 0.011) and UT-B (r = 0.7; P = 0.019) (data not shown). However, the correlation relationships between $NiCl_2$-sensitive $J_{sm\text{-}urea}$ and mRNA abundance for AQP-3 (r = 0.25; P = 0.25) and phloretin-sensitive $J_{sm\text{-}urea}$ and mRNA abundance of UT-B (r = 0.28, P = 0.19) were not significant (data not shown).

3.5 Discussion

In ruminants, UNS is an important mechanism to conserve N as plasma urea-N that passes into the GIT (particularly the rumen) can be recovered in microbial protein, thus contributing amino acids to the host animal (Fuller and Reeds, 1998). In recent years, therefore, research efforts have been directed towards improving my understanding of the nutritional and physiological mechanisms that regulate UNS. Recent important findings were: 1) that carrier-mediated, facilitative UT-B proteins were expressed in ruminal epithelia of bovine (Marini and Van Amburgh, 2003; Stewart et al., 2005) and ovine (Marini et al., 2004); 2) that adding phloretin (an inhibitor of UT-B function) to mounted ruminal epithelia in Ussing chambers reduced $J_{sm\text{-}urea}$, thus indicating that UT-B have a functional role in UNS (Stewart et al, 2005; Abdoun et al., 2010; Kiran et al., 2011); and 3) that the ruminal expression of UT-B responded to changes in levels of dietary N (Marini and Van Amburgh, 2003; Ludden et al., 2009) and concentrate (Simmons et al., 2009). In non-ruminant species, AQP-3, -7, -9, and -10 have been shown to be permeable to urea (Bagnasco, 2005); however, their role in UNS in ruminants is still unknown.

The aim of the present study, was to determine the relative functional roles of UT-B and AQP in the trans-epithelial movement of urea from blood into the rumen. The approach was to conduct measurements of total and inhibitor-sensitive $J_{sm\text{-}urea}$ using phloretin (Stewart et al., 2005; Abdoun et al., 2010) and $NiCl_2$ (Maclver et al., 2009) as inhibitors for UT-B and AQP, respectively. The results of the present study showed that $J_{sm\text{-}urea}$ was markedly reduced (by as much as 54%) when phloretin was added. These inhibitory effects of phloretin on $J_{sm\text{-}urea}$ are in agreement with other studies (Stewart et al, 2005; Abdoun et al., 2010; Kiran et al., 2011) that reported magnitudes of inhibition ranging from 20 to 50%. The addition of $NiCl_2$ also markedly reduced $J_{sm\text{-}urea}$ by 23%, thus providing the only evidence to date that AQP might play a

functional role in trans-epithelial urea transfer in ruminants. To my knowledge, Røjen et al. (2011a) is the only published study that sought to relate AQP expression in ruminal epithelia to quantitative urea transfer across the rumen. In that study, there was no relationship between AQP expression in ruminal epithelial tissue and urea extraction across the ruminal wall (measured using the venous-arterial difference technique; data reported in Kristensen et al., 2010), so the authors concluded that their results did not provide support for a role of AQP in regulating urea movement across the rumen. In the present study, the results clearly show that a portion of urea flux occurs via facilitated diffusion through $NiCl_2$-sensitive AQP. When phloretin and $NiCl_2$ were added together, the extent of inhibition of $J_{sm\text{-}urea}$ was 68% and additive, thus providing strong evidence that the phloretin- and $NiCl_2$-sensititive movement of urea occurred via 2 distinct pathways.

Correlations between mRNA and protein abundance of UT-B and AQP have yielded mixed results when determining the relative roles of urea transport for each protein. Røjen et al. (2011a) observed that mRNA abundance of AQP-3, -7, and -10 were significantly up-regulated when lactating Holstein cows were fed a high N diet, while mRNA abundance of UT-B was unaffected by dietary N intake. Conversely, western blot analysis showed that there was no change in abundance of AQP proteins, while UT-B was higher in animals fed the low N diets. The authors concluded that the role of AQP in trans-epithelial urea transport is limited; however, even though mRNA and protein abundance for UT-B and AQP were assessed, the functional roles of these proteins in urea movement across the ruminal epithelia was not measured in this study. In non-ruminant species, it is known that long- and short-term regulation of AQP is affected by vasopressin levels in the circulating blood (Rojek et al., 2008); however, the signaling pathway by which AQP-3 in particular is affected in the short-term is unclear as AQP-3 is not found in the cytosol, leading researchers to believe that minor changes in protein structure allow for activation or deactivation of this membrane protein (Ishibashi et al. 1997). It is known that increases in blood osmolarity have been associated with dehydration, resulting in increases in arginine-vasopressin secretion (Robertson and Athar, 1976). Although Schurmann et al. (2014) was unable to detect significant changes in plasma osmolarity, steers fed the MGD had numerically higher plasma osmolarity concentrations than those fed the control diet. This was in agreement with past research demonstrating that with feeding fermentable carbohydrates to ruminants, ruminal osmolality increases (Owens et al., 1998). In the current study, it was

observed that a positive relationship between AQP-3 and UT-B mRNA abundance exists with total $J_{sm\text{-}urea}$; however, the mechanism by which this increased gene expression and functional adaptation occurred is unclear. Increases in AQP-3 may have been mediated through changes in plasma osmolarity due to the increase in fermentable carbohydrate in the diet, with AQP-mediated $J_{sm\text{-}urea}$ being a side effect of osmotic regulatory pathways due to the primary role of AQP in the maintenance of osmotic balance.

It is well-established that the entry of urea from blood into the rumen increases when ruminants are fed diets that are high in ruminally-fermentable carbohydrate (RFC; Huntington, 1989; Theurer et al., 2002). Because plasma urea-N concentration typically decreases when ruminants are fed diets high in RFC, Abdoun et al. (2007; 2010) suggested that the observed stimulatory effects of dietary RFC on urea transfer to the rumen could likely be attributed to increased permeability of the ruminal epithelia to urea in response to increasing ruminal concentrations of SCFA. As increasing concentrations of SCFA are indicative of active fermentational activities in the rumen, greater rates of urea transfer will coincide with rising demands for N to support microbial growth (Abdoun et al., 2007; 2010). However, to my knowledge, no published studies have investigated the rate of functional adaptation in ruminal urea transfer when ruminants are exposed to diets high in RFC. Also, it is unknown if functional adaptation involves changes in UT-B and AQP function. My results showed that there was a linear increase in total $J_{sm\text{-}urea}$ as the length of exposure to dietary concentrate increased, with a 25% increase in $J_{sm\text{-}urea}$ by d 21. These results confirm that feeding more concentrate to increase ruminal energy supply has stimulatory effects on urea secretion into the rumen as has been reported by others (Huntington, 1989; Theurer et al., 2002). This response has partly been attributed to a decrease in ruminal NH_3-N concentration arising from more efficient microbial sequestration of NH_3-N as more energy is available. Others (Kennedy and Milligan, 1980; Kiran et al., 2011) reported a negative correlation between ruminal NH_3-N concentration and urea-N transfer into the rumen. Also, urea-N transfer into the rumen is facilitated by bacterial urease activity which maintains a favorable concentration gradient (Rémond et al., 1996), and urease activity is negatively correlated with ruminal NH_3-N concentration (Cheng and Wallace, 1979). In the present study, I observed a quadratic decrease in ruminal NH_3-N concentration as the length of exposure to dietary concentrate increased up to 21 d, and this could have increased the ruminal epithelium's permeability to urea.

Despite the linear increase in total $J_{sm\text{-}urea}$ with exposure to dietary concentrate, I did not observe any changes in phloretin-sensitive (i.e., UT-B-mediated), $NiCl_2$-sensitive (i.e., AQP-mediated), and total inhibitor-sensitive $J_{sm\text{-}urea}$. Across treatments, it is noteworthy that total inhibitor-sensitive $J_{sm\text{-}urea}$ accounted for 80% of total $J_{sm\text{-}urea}$ with no treatment effects on G_t. With inhibitor-insensitive $J_{sm\text{-}urea}$ being highly correlated with $J_{sm\text{-}mannitol}$, it is possible that the remaining $J_{sm\text{-}urea}$ (20% of total $J_{sm\text{-}urea}$) may be attributed to para-cellular or hydrophilic movement of urea. When taken together, these data point to transcellular pathways for the passage of urea from blood into the rumen which are mediated via specific proteins (UT-B and AQP). In conjunction with my observations on inhibitor-sensitive $J_{sm\text{-}urea}$, I also observed that prolonged exposure to dietary concentrate up-regulated the mRNA expression of UT-B and AQP-3 in ruminal epithelia. In support of my findings, others have observed an increase in UT-B mRNA expression in ruminal epithelia when steers were fed a concentrate-based rather than a forage-based diet (Simmons et al., 2009) or when energy intake increased in transition dairy cows (Røjen et al., 2008). Changes in UT-B mRNA expression have also been reported with other dietary factors such as dietary N concentration (Marini and Van Amburgh, 2003), thus suggesting that these alterations in UT-B expression could be responsible for changes in urea secretion into the rumen that have been reported with various dietary manipulations (Reynolds and Kristensen, 2008). Of the AQP that are permeable to urea, only AQP-3's mRNA expression in ruminal epithelia was up-regulated with prolonged exposure to dietary concentrate. I are not aware of any published studies that have reported AQP expression in ruminal epithelia when dietary concentrate is manipulated; however, Røjen et al. (2011a) observed that the mRNA expression of AQP-3, -7, and -10 were greater in cows fed a high N diet when compared to those fed a low N diet. In the present study, there were only small, non-significant differences in N intakes across dietary treatments so it is unlikely that the changes in AQP expression that I observed could be related to N intake. In non-ruminants, AQP-3 has been shown to be capable of transporting small amounts of urea; thus, it is possible that its up-regulation in the present study could be responsible for the changes in $J_{sm\text{-}urea}$ with prolonged exposure to concentrate. My hypothesis that the increase in $J_{sm\text{-}urea}$ that has been observed in ruminants fed more fermentable carbohydrates are mediated via changes in UT-B and AQP function is supported by the increased expression of UT-B and AQP-3 in ruminal epithelia, and the positive correlations between $J_{sm\text{-}urea}$ and UT-B and AQP-3 expression. However, it should also be noted that a significant portion of

the increase in $J_{sm\text{-}urea}$ was due to paracellular urea transport. Across treatments, phloretin-sensitive and $NiCl_2$-sensitive $J_{sm\text{-}urea}$ accounted for 75 and 25%, respectively, of total inhibitor-sensitive $J_{sm\text{-}urea}$, suggesting that the functional role of UT-B in urea secretion into the rumen appears to be more quantitatively important than that of AQP.

Studies examining the role of AQP in other tissues, primarily mammalian kidney, have also noted the important functions of AQP in urea transport (Rojek et al., 2008). Zhao et al. (2006) observed that mRNA abundance of AQP-3 was significantly increased in mice after an abrupt dose of 300 μmol of urea, and a significant increase in AQP-3-mediated urea transport. They also noted that in relation to UT-A abundance, mRNA abundance for AQP-3 and UT-B were increased. This is in agreement with the current study where UT-B accounted for a greater proportion of the total $J_{sm\text{-}urea}$ than that of AQP-3, possibly due to the primary roles of AQP in water movement compared to the transport of urea. Conversely, it is thought that the marked increase in placental urea permeability from 60 to 100 days of gestation is due to the increases in AQP-3 expression (Johnston et al., 2000) in ovine placental tissues. Furthermore, Grether-Beck et al. (2012) noted that topical application of urea to human skin stimulated both UT-A and UT-B, as well as AQP-3, -7 and -9 expression. Based on these previous studies, it is clear that AQP play a role in trans-epithelial movement of urea across different types of epithelial tissues, and it appears that AQP-3 is the most active.

To my knowledge, this study is the first to demonstrate that AQP play a functional role in the trans-epithelial movement of urea from blood into the rumen, and that both UT-B and AQP account for most of the passage of urea into the rumen. This study also showed that exposure to dietary concentrate produces changes in UT-B and AQP expression in ruminal epithelia that might be responsible for the changes in urea entry that have been reported previously. The regulation of UT-B and AQP expression appears to be a major point of control for UNS, so additional research is needed to fully understand how these urea transport proteins are regulated.

Table 3.1. Nitrogen intake, and concentrations of ruminal ammonia and serum urea-N in Holstein steer calves after an abrupt change in diet fermentability.

Item	Treatment					SEM	Contrast: *P* value			
	CON	G3	G7	G14	G21		CON vs. G21	Linear	Quad	Cubic
Nitrogen intake, g/d	8.32	8.55	8.74	8.90	8.90	0.52	0.51	0.38	0.46	0.41
Ruminal NH_3-N,mg/dL	14.9	15.3	18.7	19.9	15.3	1.23	0.80	0.35	<0.01	0.03
Serum urea-N, mg/dL	6.42	6.2	4.69	5.91	5.17	0.65	0.19	0.27	0.47	0.25

Table 3.2. Serosal-to-mucosal fluxes of urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$), and tissue conductance (G_t) and short-circuit current (I_{sc}) in ruminal epithelial tissues obtained from Holstein steer calves after an abrupt change in diet fermentability.

Item	Treatment						Contrast: *P* value			
							CON vs.			
	CON	G3	G7	G14	G21	SEM	G21	Linear	Quad	Cubic
$J_{sm\text{-}urea}$, nmol/(cm^2 × h)										
Total	107.7	112.6	112.8	122.8	144.2	14.5	0.09	0.08	0.60	0.85
$NiCl_2$-insensitive[1]	77.9	79.4	74.2	106.9	108.1	12.4	0.11	0.15	0.51	0.63
$NiCl_2$-sensitive[1]	29.7	14.2	20.7	18.7	37.5	16.8	0.07	0.55	0.31	0.84
Phloretin-insensitive[2]	37.8	62.2	56.7	50.7	62.5	8.5	0.22	0.08	0.74	0.34
Phloretin-sensitive[2]	69.9	74.8	75.7	72.2	96.5	13.5	0.18	0.22	0.48	0.55
Total-insensitive[3]	27.1	38.6	26.1	33.2	58.7	5.9	0.86	0.11	0.76	0.69
Total-sensitive[3]	92.5	86.2	95.1	93.8	109.1	12.4	0.99	0.22	0.52	0.95
$J_{sm\text{-}mannitol}$, nmol/(cm^2 × h)	15.8	17.0	17.6	19.5	19.8	1.63	0.14	0.06	0.53	0.81
G_t, mS/cm^2	3.46	3.38	2.80	3.27	3.20	0.29	0.37	0.61	0.23	0.41
I_{sc}, mA	11.7	17.5	17.5	10.0	13.9	2.63	0.32	0.50	0.64	0.02

[1]$NiCl_2$-insensitive $J_{sm\text{-}urea}$ equal to flux measurement after addition of $NiCl_2$; $NiCl_2$-sensitive $J_{sm\text{-}urea}$ equal to the inhibitable portion subtracted from the total flux.

[2]Phloretin-insensitive $J_{sm\text{-}urea}$ equal to flux measurement after addition of phloretin; phloretin-sensitive $J_{sm\text{-}urea}$ equal to the inhibitable portion subtracted from the total flux.

[3]Total-insensitive $J_{sm\text{-}urea}$ equal to flux measurement after both $NiCl_2$ and phloretin were added to chamber; total sensitive $J_{sm\text{-}urea}$ equal to the inhibitable portion from both $NiCl_2$ and phloretin, subtracted from the total flux.

Table 3.3. The mRNA abundance (fold-change relative to control) for UT-B and AQP in ruminal epithelial tissues obtained from Holstein steer calves after an abrupt change in diet fermentability.

	Treatment						Contrasts			
Item	CON	G3	G7	G14	G21	SEM	CON vs. G21	Linear	Quad	Cubic
AQP-3	1.00	1.55	2.40	4.47	4.68	0.72	<0.01	<0.01	0.45	0.47
AQP-7	1.00	0.03	0.04	0.13	0.15	0.29	<0.01	0.20	0.12	0.18
AQP-10	1.00	0.13	0.11	-0.02	0.01	0.32	<0.01	0.09	0.17	0.38
UT-B	1.00	1.38	1.35	2.44	3.02	0.52	<0.01	<0.01	0.85	0.78

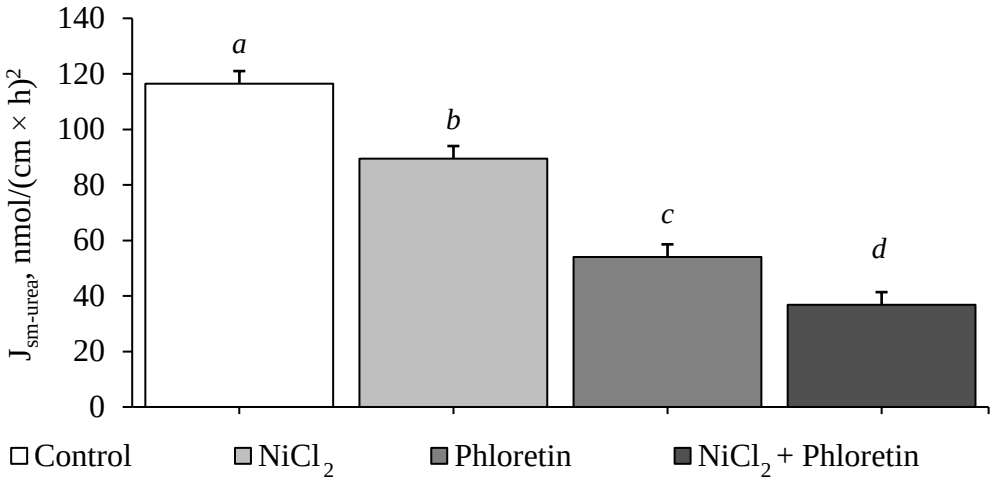

Figure 3.1. Control, $NiCl_2$-insensitive, and phloretin-insensitive and total insensitive $J_{sm\text{-}urea}$ across bovine ruminal epithelium collected from Holstein steer calves after an abrupt change in diet fermentability. Bars with different superscripts differ ($P < 0.05$).

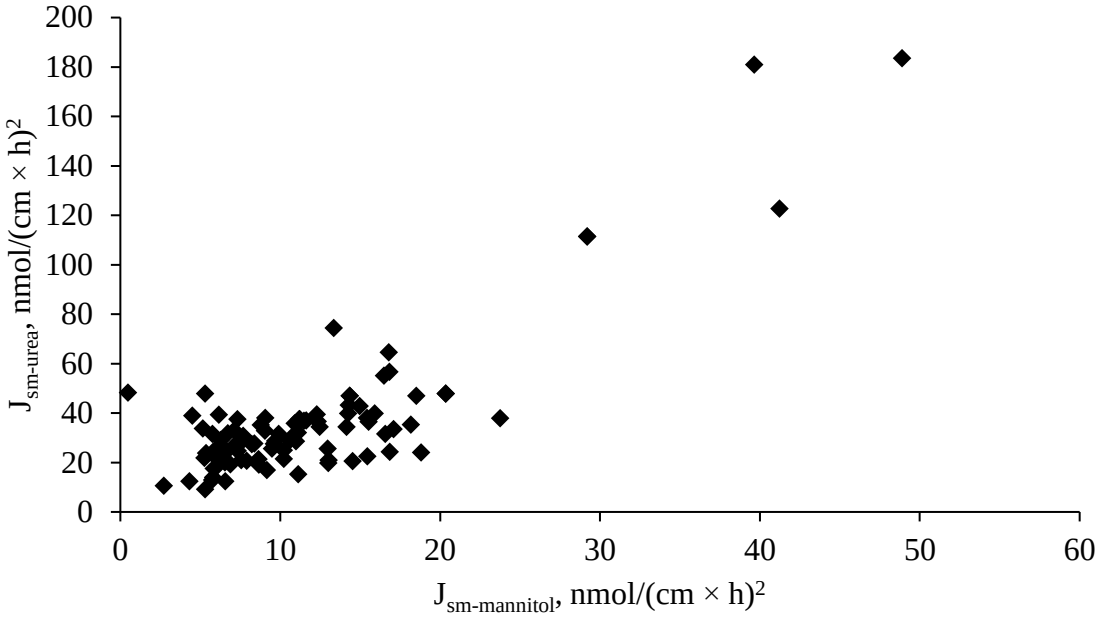

Figure 3.2. Relationship between $J_{sm\text{-}mannitol}$ and total inhibitor-insensitive $J_{sm\text{-}urea}$ in ruminal epithelia collected from Holstein steer calves after an abrupt change in diet fermentability (r = 0.89; $P < 0.001$).

CHAPTER 4. SEROSAL-TO-MUSOSAL UREA FLUX IS MEDIATED VIA $NiCl_2$-SENSITIVE PATHWAYS IN THE ISOLATED BISON (*Bison bison)* AND BEEF (*Bos taurus*) RUMINAL EPITHELIUM OBTAINED FROM ANIMALS FED BACKGROUNDING AND FINISHING DIETS.

4.1 Abstract

The objective of the study was to determine the effect of an acute dose of NH_3 on total and aquaporin (AQP)-mediated urea flux across the ruminal epithelium in Plains bison bulls (BIS) and Angus cross bulls (BEEF). Twenty-three BIS and twenty-three BOV were blocked by BW and randomly assigned to either a backgrounding (50% barley silage, 40% barley grain, and 10% mineral and vitamin supplement) or finishing (15% barley silage, 75% barley grain, and 10% mineral and vitamin supplement) diet with 3 bulls per pen. Two bulls per pen were killed each week and ruminal epithelial tissue was collected from the caudal dorsal blind sac and mounted in Ussing chambers under short-circuit conditions. The serosal buffer contained 7 m*M* urea and was adjusted to a pH of 7.4 while the mucosal buffer did not contain urea but included either 0 or 7 m*M* $(NH_4)_2CO_3$ and was adjusted to a pH of 6.2. Aquaporins were inhibited through the addition of 1m*M* $NiCl_2$ to both serosal and mucosal buffers. The serosal-to-mucosal fluxes of urea ($J_{sm\text{-}urea}$) and mannitol ($J_{sm\text{-}mannitol}$) were measured using ^{14}C-labelled urea and ^{3}H-labelled mannitol, respectively, with $J_{sm\text{-}mannitol}$ being used as an indicator of hydrophilic transport. Ruminal NH_3 was not affected by species ($P = 0.60$) or diet ($P = 0.27$) while PUN tended ($P = 0.055$) to be greater for BIS (12.5 mg/dL) than BOV (10.8 mg/dL), but was not affected by diet ($P = 0.22$). The $J_{sm\text{-}urea}$ tended to decrease with addition of $NiCl_2$ ($P = 0.065$), while mucosal NH_3 had no effect on $J_{sm\text{-}urea}$ ($P = 0.41$). $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were not affected by species ($P = 0.41$) or dietary treatment ($P = 0.29$). These results indicate that AQP may play a role in urea transport across the ruminal epithelium in both BIS and BOV bulls.

4.2 Introduction

Urea recycling is an important nitrogen salvage mechanism in ruminants, and simple manipulation of the diet, such as varying the dietary CP supply among days (Kiran et al., 2011) or increasing fermentability (Huntington and Archibeque, 1999) can increase recycling to the gastrointestinal tract. It has been suggested that bison (*Bison bison*) may have a greater capability to utilize the available N compared to other bovine species like cattle (*Bos taurus*). For

example, Deliberto (1993) observed that bison fed low protein diets (< 6% CP) had greater diet digestibility than Hereford cattle fed the same diet. Despite a lower DMI, bison maintain greater ruminal ammonia N and serum urea N concentrations when compared beef cattle (Peden, 1972; Peden et al., 1974). While it has been suggested that bison are more efficient with N conservation than beef cattle, it is unclear if bison retain this advantage when fed conventional backgrounding and finishing diets (Koch et al., 1993) and the mechanisms explaining as to why they are able to conserve N more efficiently than cattle has not been explored.

A potential explanation for the improved N efficiency for bison relative to cattle may be, in part, due to greater urea recycling to the gastrointestinal tract. Transepithelial urea flux is mediated by urea transporter B (UT-B; Abdoun et al., 2005), aquaporins (Walpole et al., 2015), and passive diffusion (Abdoun et al., 2010). It is clear that SCFA stimulate urea secretion across the rumen, which partly explains why increasing diet fermentability improves urea-N recycling to the GIT (Abdoun et al., 2010; Lui et al., 2015). On the other hand, increasing ruminal ammonia concentration reduces rate of urea re-entry into the reticulo-rumen in both wild and domesticated ruminants (Kennedy and Milligan, 1978). While there have been substantial advancements in the knowledge regarding regulation of UT-B (Abdoun et al., 2005, Kiran et al., 2010, Lui et al., 2015), little is known about AQP (Walpole et al., 2015). Furthermore, no studies known to me at the current time have compared differences among these species.

The hypothesis of the present study was that a greater rate of urea transport across the ruminal epithelium and that this transport would be regulated via AQP. I further hypothesized that the addition of NH_3 would inhibit AQP-mediated $J_{sm\text{-}urea}$. The objectives of the current study were to determine the functional role of AQP in transepithelial urea flux as affected by diet fermentability and ammonia concentration.

4.3 Materials and Methods

4.3.1 Animal husbandry and experimental design

All procedures performed in this study were preapproved by the University of Saskatchewan Animal Research Ethics Board (protocol 2011034). Twenty-three plains bison bulls (*Bison bison*) were obtained from Elk Island National Park (Alberta, Canada) and 23 Angus crossbred cattle (*Bos taurus*) were obtained from the Kinsella Ranch at the University of Alberta (Alberta, Canada). All animals were transported to the Goodale Farm at the University of

Saskatchewan (Saskatoon, Saskatchewan, Canada). Upon arrival, all animals were ranked by BW and allocated into 8 pens (n = 3/pen; consisting of 2 treatment animals and 1 companion animal; the latter which was not slaughtered). For both bison and beef, one companion animal was used twice. Each pen was randomly allocated to one of two treatments by species, consisting of either a backgrounding (BGK) or finishing (FIN) diet (Table 4.1). A gradual diet transition was provided and animals on the BGK and FIN diets received was staggered (2 intermediary diets over 14 d for BGK and 7 intermediary diets over 27 d for FIN) such that animals on either the BGK or FIN treatments received their final diet for 30 d. Animals were fed daily at 1000 h and refusals were collected, weighed, and analyzed for DM content every 14-d. Animals were weighed on a single day 4 and 2 weeks prior to slaughter, and immediately before being transported to a local abattoir for slaughter, leaving the facility at 0700 h. Upon arrival at the abattoir (Warmen, SK, Canada), all animals were killed by captive bolt stunning followed by exsanguination. Only 1 animal was killed per day to facilitate measurements of ex vivo transepithelial flux.

4.3.2 Measurement of reticulo-rumen pH

Reticulo-rumen pH was measured using an orally doseable pH meter described by Penner et al. (2009; Dascor, Escondido, CA). The pH device was administered orally 14 d prior to slaughter, and was set to take a measurement every 10 min. The pH meter was recovered after killing and the location within the reticulo-rumen was recorded. Prior to dosing and after recovery of the pH meter, mV readings in standard pH buffers (4.0 and 7.0) were recorded and used to derive linear equations to convert mV readings to pH values. These equations (prior to dosing and after recovery) were used together by incorporating a linear offset to convert logged data to pH. Only data from the last 7 d of measurement were used to calculate the minimum, mean, and maximum pH values. The duration and area (AREA) that pH was below 5.5 were also determined.

4.3.3 Blood Collection and Analysis

Blood was collected 14 d prior to slaughter. Samples were collected from the jugular vein into 2 separate tubes, one containing sodium-heparin (148 IU sodium-heparin; Becton Dickinson, Franklin Lakes, NJ) for plasma and the second into a EDTA-coated vacutainer® for serum (Becton Dickinson). Samples were placed on ice until centrifugation at 1800g for 15 min at 4°C and then stored at -20°C until analysis of plasma glucose and insulin, and serum non-

esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), and urea. Plasma glucose was determined using a glucose oxidase-peroxidase enzyme assay (PGO Enzyme Preparation; Sigma-Aldrich Co., St. Louis, MO). The change in colour was measured at 450 nm on a SpectraMax Plus Microplate Reader (Molecular Diagnostics, Inc., Sunnyvale, CA, USA), and is proportional to the original plasma glucose concentration. Plasma insulin was measured using a bovine-specific insulin ELISA kit (Mercodia, Uppsala, Sweden). BHBA was analyzed through the addition of 3-hydroxybutyrate-dehydrogenase to catalyze the reaction of BHBA to acetoacetate with an equimolar amount of NAD being reduced to NADH. The absorbance was then read at 340 nm on a microplate reader, with the increase in absorbance is directly proportional to the BHBA content of the original sample. A commercial kit was used to determine the concentration of NEFA (HR series NEFA-HR2; Wako Chemical, Atlanta, GA). Serum was analyzed for urea nitrogen using the diacetylmonoxime method (Stanbio, procedure 0580, Boerne, TX)

4.3.4 Measurement of $Na^{2+}$and urea transport in Ussing Chambers

Within 5 minutes of exsanguination, the digestive tract was removed from the abdominal cavity and a 300-cm^2 piece of rumen wall was taken from the caudal-dorsal sac, washed in a physiological buffer solution and stripped of the muscle layer. The epithelium was then placed in a physiological buffer solution maintained at 38°C and transported the laboratory (approximately 30 min). The buffer was gassed with 95% O_2:5% CO_2 for 60 min prior to transport. The physiologic buffer solution contained (m*M*): 15.6 NaCl, 5.5 KCl, 1.0 $CaCl_2$, 1.3 $MgCl_2$, 0.6 NaH_2PO_4, 2.4 Na_2HPO_4, 1.0 L-glutamine, 10.0 HEPES-free acid, 24.0 $NaHCO_3$, 5.0 Na-D/L-Lactate, 20.0 Na-acetate, 10.0 acetic acid, 15.0 Na-propionate, 5.0 Na-butyrate, and 60 mannitol.

Upon arrival at the laboratory, ruminal epithelia were cut into strips and placed between two halves of an Ussing chamber with an exposed surface area of 1.43 cm^2 (n = 6, for measurement of urea flux) or 3.14 cm^2 (n = 4, for measurement of Na^+ flux). On each the mucosal and serosal sides of the ruminal epithelia were buffer solutions (10 mL for urea flux and 15 mL for Na flux) circulated by gas lift (95% O_2:5% CO_2) and temperature was maintained at 38.5°C using a water jacket. Buffer solutions were the same as previously described except that antibiotics (penicillin G sodium salt, 60 $mg \cdot L^{-1}$; kanamycin sulphate, 100 $mg \cdot L^{-1}$; and flurocytosine 50 $mg \cdot L^{-1}$) and 1 m*M* phenyl-phosphorodiamidate (urease inhibitor; Alfa Aesar,

Ward Hill, MA) were included. Serosal and mucosal buffer solutions were adjusted to 7.4 and 6.2, respectively using 1 m*M* NaOH or 1 gluconic acid (48%) to mimic in *vivo* conditions.

4.3.5 Measurement of the serosal-to-mucosal urea ($J_{sm\text{-}urea}$) and mannitol flux ($J_{sm\text{-}mannitol}$).

For the determination of $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, a computer-controlled voltage-clamp (VCC MC6; Physiologic Instruments) was used to incubate tissues under short-circuit conditions and for the measurement of tissue conductance ($\mathbf{G_t}$) as described by Doranalli et al. (2011). $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were measured in parallel using 6 individual epithelia. Epithelia were assigned to 1 of 3 treatments (CON vs. NH_3 vs. $NiCl_2$; $n = 2$) over 2 flux periods such that the treatments were balanced for mean G_t (Doranalli et al., 2011). In order to measure $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$, 26.25 kBq of ^{14}C-labelled urea (Perkin-Elmer) and 74 kBq of ^{3}H-labelled mannitol (Perkin-Elmer) were added to the serosal buffer solutions along with non-labelled urea and mannitol to achieve a final concentrations of 7 m*M* and 1 m*M*, respectively. This approach created a urea gradient to mimic the physiologic conditions, where urea is not normally found in rumen fluid (Kiran et al., 2011). A mannitol gradient was also imposed. Samples from the mucosal side (500 µL) were taken after a 45-minute radioactivity equilibration period and at the beginning and end of each of three 30-minute flux periods. An equivalent amount of fresh buffer was replaced to minimize changes associated with hydrostatic pressure. Samples from the serosal side (100 µL) were collected before the first and after the last flux period for calculation of specific radioactivity using a scintillation counter (Perkin Elmer). Treatment combinations for period were as follows; CON (no inhibition), NH_3 (mucosal addition of $(NH_4)_2CO_3$ to result in a final concentration of 7 m*M* NH_3/NH_4^+), and Ni^{2+}, a known inhibitor of aquaporins (Maclver et al., 2009; serosal and mucosal addition of $NiCl_2$ resulting in a final concentration of 1 m*M*). During the period 2, inhibitors were combined resulting in treatment combinations CON, NH_3 + $NiCl_2$, and $NiCl_2$ + NH_3. A 15-min inhibitor equilibration period was provided before the start of each 30-min flux period. All chemicals (reagent grade), $NiCl_2$ and antibiotics were obtained from Sigma-Aldrich unless otherwise stated.

4.3.6 Measurement of Na flux.

Tissues used for measurement of $J_{sm\text{-}Na}$ (n = 2) and $J_{ms\text{-}Na}$ (n = 2) were incubated under short-circuit conditions as described by Aschenbach et al., (2000) using a computer controlled voltage

clamp device. Epithelia were allowed 20 minutes for the stabilization of electrophysiology and tissue conductance was recorded. Tissues were then paired by G_t (not differing by more than 20%) and randomly assigned to either $J_{ms\text{-}Na}$ or $J_{sm\text{-}Na}$, in order to determine $J_{net\text{-}Na}$ ($J_{ms\text{-}Na}$ - $J_{sm\text{-}Na}$). After treatment assignment, 80 kBq of ^{22}Na was added to either the mucosal or serosal buffer, and was allowed to equilibrate for 45 minutes as outlined by Sehested et al. (1996). Samples (100 µL) were collected from the side where radioactivity was added at the beginning and end of the experiment. Samples (500 µL) from the opposite side were collected with 1 h intervals and fresh buffer solution was replaced. Radioactivity was measured using a gamma counter.

4.3.7 Ruminal Fluid Collection and Analysis

Rumen digesta were collected at slaughter and strained through 2 layers of cheesecloth to obtain ruminal fluid. Two 10-mL samples were preserved with either 2 mL of metaphosphoric acid (25% wt. /v) for short-chain fatty acid (SCFA) analysis or 2 mL of 1% sulfuric acid for rumen NH_3-N analysis. Samples were stored at -20°C. The SCFA were determined using gas chromatography (Khorasani et al., 1996), and rumen NH_3-N was determined using a phenol-hypochlorite assay (Broderick and Kang, 1980).

4.3.8 Calculations and Statistical Analysis

Flux measurements were calculated as described by Gäbel et al. (1989). Total $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ are flux measurements obtained from chambers that had no inhibitor added to the incubation buffer. Inhibitor-insensitive $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were measured from chambers with inhibitors added to the incubation buffer. Inhibitor-sensitive flux was calculated as total flux – inhibitor-insensitive flux. $J_{net\text{-}Na}$ was calculated by the difference between $J_{sm\text{-}Na}$ and $J_{ms\text{-}Na}$.

All statistical analysis was conducted using the mixed procedure of SAS (version 9.3; SAS Institute, Inc., Cary, NC). For in vivo measurements, the model consisted of species (BIS vs. BEEF), diet (BKGD vs. FIN), and species × diet as fixed effects. The pen (experimental unit) and block were included as random effects. Where ex vivo treatments were applied, the model was similar as above with the exception that the in vitro treatment was included as a sub-plot factor (fixed effect) in a split-plot design and tissue replicate within pen as the random effect. Regression analysis was performed using the PROC REG of SAS (SAS Institute, Cary, NC). In all cases significance was declared when $P < 0.05$.

4.4 Results

4.4.1 Body weight and dry matter intake (DMI)

Body weight at the start and end of the study differed (species, $P < 0.001$; Table 4.2) between BEEF and BIS averaging 454 and 547 kg for BOV and 335 and 389 for BIS, respectively. However, there were no effects of diet or interaction between species and diet ($P > 0.05$). Whether reported in kg/d ($P = 0.07$) or as a percentage of BW ($P = 0.07$), DMI tended to be lower for BIS than BOV.

4.4.2 Ruminal Fermentation

Interactions between species and diet were detected for minimum ($P = 0.003$) and mean reticulo-rumen pH ($P = 0.004$) where the magnitude for the reduction for the BKGD and FIN diets was greater for BIS than for BOV (Table 4.3). In fact, for mean pH, values were not different for BEEF between BKGD and FIN while for BIS the mean pH was nearly 0.5 pH units lower during FIN than BKGD. Despite lower mean pH, the duration that rumen pH was below 5..5 was greater in BEEF compared to BIS ($P = 0.028$), but was not affected by diet ($P = 0.36$).

Ruminal ammonia concentration did not differ between species ($P = 0.68$) but, FIN bulls had greater ruminal ammonia concentration (18.1 vs. 5.2 mg/dL, $P < 0.001$; Table 4.3) than BKGD. Total SCFA concentration did not differ among species or diet ($P \geq 0.22$) averaging 94.4 m*M*. For acetate, the molar proportion was 5.2 percentage units lower for BOV than BIS ($P = 0.003$) and was 9.2 percentage units lower for bulls fed FIN compared to BKGD ($P < 0.001$). Correspondingly, the molar proportion of propionate was greater for BEEF than BIS ($P = 0.006$) and greater for FIN than BCKD ($P < 0.001$). The molar proportions of butyrate, isobutyrate, and isovalerate were not affected by species or diet. However, an interaction ($P < 0.001$) was detected for valerate where during backgrounding BEEF and BIS did not differ (1.1 vs. 1.1%) with BEEF increasing to a greater extent than BIS for FIN (3.3 vs. 1.8%).

4.4.3 Circulating metabolites

BIS had greater concentrations of serum BHBA ($P < 0.001$), NEFA ($P = 0.002$), and plasma glucose ($P < 0.001$; Table 4.4) than BEEF. Additionally, FIN bulls had concentrations of serum NEFA that were less than bulls fed the BKGD diet ($P = 0.04$). Plasma urea nitrogen concentration was greater for BIS than BEEF ($P = 0.014$), but PUN was not affected by diet ($P = 0.16$).

4.4.4 Urea and sodium flux

The $J_{ms\text{-}Na}$ was not different between species or dietary treatment (Table 4.5), however , $J_{sm\text{-}Na}$ was greater in BIS fed the BKGD diet compared to FIN, but $J_{sm\text{-}Na}$ in beef bulls was similar between both dietary treatments (species diet; $P = 0.021$). Bison fed FIN tended (species × diet, $P = 0.058$) to have greater $J_{net\text{-}Na}$ compared to BIS fed BKGD; however, the $J_{net\text{-}Na}$ did not differ for BEEF regardless of diet.

Across treatments, the in vitro addition of 1 mM $NiCl_2$ reduced $J_{sm\text{-}urea}$ ($P = 0.009$) while the addition of 7 mM NH_4Cl had no effect on $J_{SM\text{-}urea}$ ($P = 0.81$; Figure 4.1). Addition of $NiCl_2$ and NH_4Cl in combination also decreased $J_{sm\text{-}urea}$ ($P = 0.004$; Figure 4.1), but was not different from that of $NiCl_2$ alone. Bulls fed FIN tended to have greater $J_{sm\text{-}urea}$ than bulls fed BKGD ($P = 0.057$; Table 4.5), while species had no effect on $J_{sm\text{-}urea}$ ($P = 0.54$). Neither diet nor species affected the inhibitor-sensitive (i.e. $NiCl_2$- or $(NH_4)_2CO_3$-sensitive components) $J_{sm\text{-}urea}$ ($P > 0.1$). Tissue conductance (species × diet $P = 0.034$) did not differ between BEEF and BIS when fed BKGD but increased for BEEF and decreased for BIS when fed FIN. Short-circuit current (species × diet $P = 0.033$) was greater during FIN than BKGD for BEEF but not for BIS.

Despite the lack of effect for ammonia ex vivo, ruminal NH_3 concentration at killing and $J_{sm\text{-}urea}$ were not correlated for BIS ($R^2 = 0.1658$, P =0.13) or BEEF ($R^2 = 0.0029$, P =84; Figure 4.2). Regression analysis between $J_{sm\text{-}urea}$ for BIS ($R^2 = 0.1017$, $P = 0.086$) and BEEF ($R^2 = 17$, $P = 0.019$, Figure 4.3) and tissue conductance (G_t) depicted a negative relationship. A similar trend was noted for the relationship between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ for BIS ($R^2 = 0.6648$, P = 0.0004) but was not seen for BEEF ($R^2 = 0.395$, $P = 0.49$, Figure 4.4).

4.4 DISCUSSION

Bison have long been thought to be more efficient with regards to urea-N recycling than cattle when fed diets high in forage and low in CP (Peden, 1972; Peden et al., 1974; DeLiberto, 1993). Moreover, there is evidence to support that digestibility of diets varying in forage content are greater for BIS than BEEF (Koch et al., 1993). This may suggest that part of the beneficial response for N efficiency for BIS could be related to improved digestibility along with urea-N recycling. That said, the underlying mechanisms for urea recycling in BIS have not been evaluated and it is not known whether they differ from BEEF. The objective of this study was to determine whether BEEF and BIS differed with respect to ruminal fermentation and whether

AQP-mediated and ammonia-inhibited urea flux differed among species and when fed BKGD and FIN diets.

In the current study, the intention was to manage BIS and BEEF bulls similarly; however, it is important to note that initial and final BW were greater for BEEF than BIS. As a result, DMI tended to be greater for BEEF than BIS whether reported in kg/d or as a percentage of BW. Greater DMI for BEEF than BIS has been previously reported by DeLiberto et al. (1993) and Koch et al. (1993). Thus, although not measured, dietary N intake was expected be greater for BEEF than BIS. These points are relevant as factors known to affect transepithelial flux include ruminal pH, SCFA concentration, and ammonia concentration (Lapierre and Lobley, 2001); all of which are related to DMI. I observed that BIS had greater mean reticulo-ruminal pH when fed BKGD (6.70) than FIN (6.22) with pH during FIN for BIS being less than for BEEF. The observation that reticulo-ruminal pH for BIS was less than BEEF during finishing is surprising given the differences for DMI, but may suggest that factors involved in reticulo-ruminal pH regulation differ between species. Regardless of the differences, mean pH values observed are within the range suggested to maximize transepithelial urea flux (Abdoun et al., 2009). Unlike for reticulo-ruminal pH, total SCFA concentration did not differ between BIS and BEEF and furthermore did not differ between BKGD and FIN diets. Recent studies have demonstrated that the transepithelial urea-N flux is enhanced by SCFA; however, given that concentration did not differ between species or diet, it is unlikely that potential differences in transepithelial urea-N flux would be related to SCFA concentration. I also observed that ruminal ammonia-N concentration did not differ between BIS and BEEF, while markedly greater ruminal ammonia-N concentrations were observed for FIN (18.1 mg/dL) than BKGD (5.2 mg/dL). It is not clear why FIN bulls had greater ruminal ammonia-N, especially considering: 1) the greater inclusion rate of barley grain in the FIN diet relative to BKGD; 2) that dietary CP was similar between the FIN and BKGD treatments, and 3) that PUN concentration did not differ by diet. Although ruminal ammonia concentration did not differ by species, BIS had greater PUN than BEEF, regardless of diet. Past research has shown that transepithelial urea flux is positively related to PUN concentration (Sunny et al., 2007). Collectively, these data indicate that of the in vivo variables, measured, that may promote or inhibit transepithelial urea-N recycling, only PUN was greater for BIS relative to BEEF. Greater PUN may provide a means to promote transepithelial urea flux by maintaining a greater concentration gradient.

To evaluate whether BIS and BEEF differed in the inherent capability for transepithelial urea flux, the current project evaluated the total flux and inhibition of flux using isolated ruminal epithelia mounted in Ussing chambers. Results indicate that there were no differences in the rate of $J_{sm\text{-}urea}$ between BIS and BEEF. Thus, it does not appear that the differences in N use efficiency between BIS and BEEF is related to total trans-epithelial transport of urea. Regarding the pathways involved in $J_{sm\text{-}urea}$, I observed that AQP-mediated $J_{sm\text{-}urea}$ ($NiCl_2$-sensitive) contributed 17.3 % of the total $J_{sm\text{-}urea}$. This is in agreement with previous work where $NiCl_2$-sensitive $J_{sm\text{-}urea}$ accounted for 23% of the total $J_{sm\text{-}urea}$ in the bovine ruminal epithelium (Chapter 3). Although few studies have been conducted to examine the functional roles of AQP in the ruminal epithelium, some research has evaluated the relative expression of AQP and UT-B in relation to protein abundance (Røjen et al., 2011a). In that study, it was observed that when dairy cattle were fed diets containing two levels of CP, animals fed the high CP diets had higher levels of mRNA abundance of AQP -3, -7, and -10 while protein abundance of AQP3, and 7 were unaffected by dietary treatment while protein abundance of AQP8 was increased on the high CP diet. Feeding of high CP diets results in a higher BUN that can result in a higher transfer of urea-N into the gastrointestinal tract (Sunny et al. 2007). Similarly, increasing dietary carbohydrate fermentability has been shown to increase urea-N efficiency (Chibisa et al., 2015) as well as increase $J_{sm\text{-}urea}$ (Walpole et al., 2015). Results from the present study support the latter finding where $J_{sm\text{-}urea}$ tended to be greater for FIN than BKGD bulls. Ultimately, present results demonstrate that $NiCl_2$-sensitive AQP pathways are important in the transport of urea across the ruminal epithelium in both bison and beef bulls.

In addition to AQP, UT-B are known transporter for $J_{SM\text{-}urea}$ in the ruminal epithelium. In the present study, I did not evaluate the contribution of UT-B but assessed the relationship between $J_{sm\text{-}urea}$ and Gt and $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ where I observed a significant negative correlation between $J_{sm\text{-}urea}$ and Gt (Figure 4.3), as well as a tendency for a negative relationship between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ (Figure 4.4). Increasing Gt and $J_{sm\text{-}mannitol}$ have been linked with decreases in ruminal epithelial barrier function (Schurmann et al., 2014), resulting in an increase in para-cellular $J_{sm\text{-}urea}$ (Walpole et al., 2015). However in the current study it is clear that as paracellular transport increased, the total flux of urea across the ruminal epithelium was depressed.

There is discrepancy regarding the inhibitory role of ammonia on transepithelial urea flux. In previous studies by Doranalli et al. (2012) and Muschler et al. (2010) it was noted that increased ruminal NH_3 concentration resulted in a reduction in $J_{sm\text{-}urea}$, while a positive relationship was noted between $J_{sm\text{-}urea}$ and both $J_{sm\text{-}mannitol}$ and Gt. In the present study ruminal NH_3 at the time of killing was not related to $J_{sm\text{-}urea}$. This may be attributed to the moderately and high levels of fermentable carbohydrates fed in this study. Theurer et al. (2002) noted that when dietary starch fermentability was increase, it caused a decrease in ruminal NH_3 as well as a higher rate of urea-N recycled back to the GIT. This high rate of microbial protein synthesis, due to the availability of ruminally fermentable starch likely produced an environment where ruminal NH_3 was limiting, producing a strong osmotic gradient towards $J_{sm\text{-}urea}$. Although the ruminal NH_3 concentration in finishing bulls was greater than for backgrounding bulls it must be noted that bulls were fed at 1000 h; however, on the sample collection day, bulls left for the abattoir at 0700 h. It is known that meal size is greatest after the delivery of fresh feed and that changes in feeding time can have impacts on ruminal fermentation as well as animal performance (Schwartzkopf-Genswein et al., 2004). Due to the physical size of these bulls and the location of the research facility, all bulls were held off feed for 3 hours prior to being killed. In the case of the finishing bulls, that tended to have higher $J_{sm\text{-}urea}$ ex vivo, it is likely that the high ruminal NH_3 is due to an adaptive response to the ruminal epithelium in combination with being withdrawn from feed prior to slaughter. Thus, it is speculated that since bulls were not provided fresh feed, there was a lack of energy available in the rumen to promote ruminal fermentation and either continued trans-epithelial urea transport, amino acid deamination, or a combination of them occurred to result in greater ammonia at the time of killing. Although Lu et al. (2014) noted that a mucosal addition of 2.5 mM NH_3 ex vivo caused a reduction in $J_{sm\text{-}urea}$ and $J_{ms\text{-}urea}$, they did not outline the ruminal conditions prior to slaughter as well as to if animals were held off feed prior to being killed for tissue collections.

Sodium transport in the ruminal epithelium has been described previously (Gäbel et al., 1989, 1991; Sehested et al., 1999; Etchmann et al., 2009; Shen et al., 2012). Na^+ absorption across the ruminal epithelium occurs through highly regulated mechanisms. Part of the Na^+ flux occurs through sodium hydrogen exchanger-1 and -3, where transport of Na^+ into the cytosol occurs in exchange for H^+ as a method to regulate intracellular pH. Given that SCFA stimulate $J_{SM\text{-}urea}$ (Abdoun et al., 2010), that SCFA absorption is linked with Na^+ transport (Sehested et al.,

1991), and that NH_4^+ transport can alter Na^+ transport (Lu etl al., 2015), This project evaluated whether diet and species differed in Na^+ flux. The main finding was that although $J_{MS\text{-}Na}$ did not differ between species or diets, the $J_{sm\text{-}Na}$ was greater for BIS when fed BKGD than for BEEF regardless of diet and greater than when BIS were fed FIN than BKGD. $J_{sm\text{-}Na}$ is thought to be a paracellular process (Gäbel et al., 1989) and may indicate that the ruminal epithelium of BIS when fed the BKGD diet was 'leaky'. It is puzzling why the ruminal epithelium for BIS would be more 'leaky' when fed the BKGD diet than FIN as reticulo-ruminal pH was lower, however tissue G_t supports this finding. It is possible, that the lower DMI for BIS on the BKGD treatment may have been a partial causative factor for this response as reductions in DMI have been reported to decrease total gastrointestinal barrier function in beef cattle (Zhang et al., 2013) and anecdotal observations from the present study suggest that after removal of the first BIS for each block, there was a reduction in feed intake. However, given that DMI was recorded on a weekly basis, no confirmation of this statement can be made.

In conclusion, it is clear that BEEF have greater DMI than BIS, but BIS may be more susceptible to low reticulo-ruminal pH when fed highly fermentable diets. In addition, BIS had greater PUN concentration than BEEF, regardless of diet but these differences in ruminal fermentation did not translate into differences for $J_{sm\text{-}urea}$ between species. My data does, however, suggest that the activity of AQP increases with increasing diet fermentability and that ammonia is not a potent inhibitor of $J_{sm\text{-}urea}$ ex vivo.

Table 4.1. Ingredient and nutrient composition of the transition diets used to adapt *Bison bison* and *Bos taurus* bulls to the backgrounding and finishing diets.

	Backgrounding (BG) diets			Finishing (FIN) diets							
Item	BG1	BG2	BG3	FIN1	FIN2	FIN3	FIN4	FIN5	FIN6	FIN7	FIN8
Inclusion rate, % DM											
Barley silage	65	55	45	65	55	45	35	25	20	15	10
Barley grain	25	35	45	25	35	45	55	65	70	75	80
Backgrounding pellet[1]	10	10	10	-	-	-	-	-	-	-	-
Finishing pellet[2]	-	-	-	10	10	10	10	10	10	10	10
Days fed	1 to 7	8 to 14	15 to slaughter	1 to 4	5 to 8	9 to 12	13 to 16	17 to 20	21 to 24	24 to 27	28 to slaughter
Nutrient composition, % DM											
CP	14.0	14.1	14.1	14.0	14.0	14.1	14.2	14.3	14.3	14.4	14.4
NDF	40.9	37.1	33.4	40.9	37.2	33.4	29.7	25.9	24.0	22.1	20.3
Starch	23.2	27.8	32.5	22.9	27.6	32.3	36.9	41.6	43.9	46.3	48.6
Ca	0.8	0.7	0.7	0.8	0.8	0.8	0.7	0.7	0.7	0.7	0.6
P	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

1Contained (% DM) ground barley grain (50.5), corn distillers grain with solubles (25), limestone (8.7), dynamate (6.8), canola meal (5.9), salt (2.1) and a trace mineral and vitamin pre-mix (1). The pellet contained (% DM) CP (13.6), crude fat (3.4), Salt (2.30), Ca (3.44), P (0.54), Mg (1.11), K (1.78), S (1.81), microminerals (mg/kg) Co (4.6), Cu (146.1), I (8.0), Fe (451.8), Mn (335.4), Se (2.26), Zn (313.7), Fl (11.3), and vitamins (IU/kg) A (40,000), D3 (15,000), and E (300).

2Contained (% DM) ground barley grain (50.5), corn distillers grain with solubles (25), limestone (8.7), dynamate (6.8), canola meal (5.9), salt (2.1) and a trace mineral and vitamin pre-mix (1). The pellet contained (% DM) CP (13.6), crude fat (3.4), Salt (2.30), Ca (3.44), P (0.54), Mg (1.11), K (1.78), S (1.81), microminerals (mg/kg) Co (4.6), Cu (146.1), I (8.0), Fe (451.8), Mn (335.4), Se (2.26), Zn (313.7), Fl (11.3), and vitamins (IU/kg) A (40,000), D3 (15,000), and E (300).

Table 4.2. Body weight (BW), dry matter intake (DMI), and average daily gain (AVG) for *Bos taurus* and *Bison bison* bulls fed either a backgrounding or finishing diet.

	Beef		Bison			*P* value		
Item[1]	Backgrounding	Finishing	Backgrounding	Finishing	SEM	Species	Diet	Species × diet
Initial BW, kg	422	419	305	296	9.1	< 0.001	0.54	0.72
Start of test BW, kg	454	453	350	319	8.0	< 0.001	0.056	0.074
Ending BW, kg	536	558	392	385	11.2	< 0.001	0.50	0.18
DMI, kg/d	13.4	13.2	10.1	8.5	2.00	0.069	0.67	0.74
DMI, % BW	2.40	3.38	1.88	2.25	0.411	0.068	0.13	0.48
DMI, % BM^{MET}	11.6	14.9	9.04	9.83	1.04	0.045	0.21	0.39
ADG, kg/d	2.02	2.24	1.38	1.36	0.196	< 0.001	0.59	0.54

[1]Data from the final week of feeding were used for statistical analysis. This equated to wk. 7 for backgrounding and wk. 9 for finishing. End of test body weight was used to calculate DMI as a percent of BW.

Table 4.3. Ruminal pH and concentrations of ammonia and short-chain fatty acids in *Bos taurus* and *Bison bison* bulls fed either a backgrounding or finishing diet.

	Beef		Bison			*P* value		
Item	Backgrounding	Finishing	Backgrounding	Finishing	SEM	Species	Diet	Species × diet
Rumen pH								
Minimum	6.11	5.89	6.36	5.66	0.085	0.86	< 0.001	0.003
Mean	6.49	6.46	6.70	6.22	0.794	0.86	0.001	0.004
Maximum	6.88	6.87	6.98	6.81	0.084	0.79	0.28	0.34
Duration pH < 5.5, min/d	132.6	194.2	52.5	73.9	47.63	0.03	0.36	0.66
Area pH < 5.5, pH × min/d	17.2	28.3	7.9	14.1	7.20	0.087	0.21	0.72
Rumen metabolites								
Ammonia-N, mg/dL	4.7	19.8	5.7	16.4	2.72	0.68	< 0.001	0.43
Total SCFA, m*M*	81.3	83.6	84.5	106.3	8.35	0.13	0.16	0.25
Acetate, mmol/100 mmol	65.1	53.6	68.0	61.1	1.66	0.003	< 0.001	0.18
Propionate, mmol/100 mmol	18.0	28.0	15.6	18.9	1.92	0.006	0.002	0.09
Butyrate, mmol/100 mmol	13.8	11.8	13.4	15.3	1.20	0.21	0.98	0.12
Isobutyrate, mmol/100 mmol	0.9	1.2	0.9	1.0	0.11	0.39	0.11	0.51
Valerate, mmol/100 mmol	1.1	3.3	1.1	1.8	0.18	< 0.001	< 0.001	< 0.001
Isovalerate, mmol/100 mmol	1.2	2.1	1.0	1.9	0.28	0.58	0.002	0.98

Table 4.4. Blood metabolites and plasma insulin for *Bos taurus* and *Bison bison* bulls fed either a backgrounding or finishing diet.

	Beef		Bison			*P* value		
Item	Backgrounding	Finishing	Backgrounding	Finishing	SEM	Species	Diet	Species × diet
BHBA, mg/dL	17.4	16.9	24.7	24.9	2.01	< 0.001	0.93	0.86
NEFA,	148.8	135.7	262.2	173.9	23.94	0.002	0.032	0.108
PUN, mg/dL	10.2	11.3	12.2	13.3	0.81	0.014	0.16	0.97
Glucose, mg/dL	73.1	72.9	86.0	87.7	3.63	< 0.001	0.83	0.79
Insulin µg/L	0.29	0.41	0.27	0.36	0.06	0.55	0.068	0.80

Table 4.5. Mucosal-to-serosal ($J_{ms\text{-}Na}$), serosal-to-mucosal ($J_{sm\text{-}Na}$), and net Na^+ flux ($J_{net\text{-}Na}$), [nmol/ (cm^2 × h)] across the isolated rumen epithelium from *Bos taurus* and *Bison bison* bulls fed either a backgrounding or finishing diet.

	Beef		Bison			*P* value		
Item	Backgrounding	Finishing	Backgrounding	Finishing	SEM	Species	Diet	Species × diet
$J_{ms\text{-}Na}$	91.0	80.9	100.1	110.0	13.0	0.12	0.82	0.59
$J_{sm\text{-}Na}$	31.8	45.7	0.72	0.52	7.8	0.004	0.65	0.021
$J_{net\text{-}Na}$	59.2	35.2	35.0	58.0	12.7	0.96	0.97	0.058

Table 4.6. Serosal-to-mucosal flux of urea and mannitol [nmol/ (cm^2 × h)], tissue conductance [mS/ (cm^2 × h)], and short-circuit current [uA/ (cm^2 × h)], in isolated epithelia from *Bos taurus* and *Bison bison* bulls fed either a backgrounding or finishing diet.

	Beef		Bison			*P* value		
Item	Backgrounding	Finishing	Backgrounding	Finishing	SEM	Species	Diet	Species × diet
$J_{sm\text{-}urea}$								
Total	27.1	33.2	24.9	30.0	4.8	0.54	0.057	0.65
$NiCl_2$ Sensitive	-2.9	4.1	5.9	12.8	7.1	0.26	0.33	0.84
NH_3 Sensitive	-0.01	8.0	-9.8	-0.8	0.06	0.41	0.74	0.73
$J_{sm\text{-}mannitol}$	61.1	45.6	68.6	110.9	207	0.85	0.95	0.89
G_t	3.55	5.04	3.31	1.84	0.98	0.063	0.98	0.034
I_{sc}	19.0	27.9	19.5	19.0	5.14	0.039	0.64	0.033

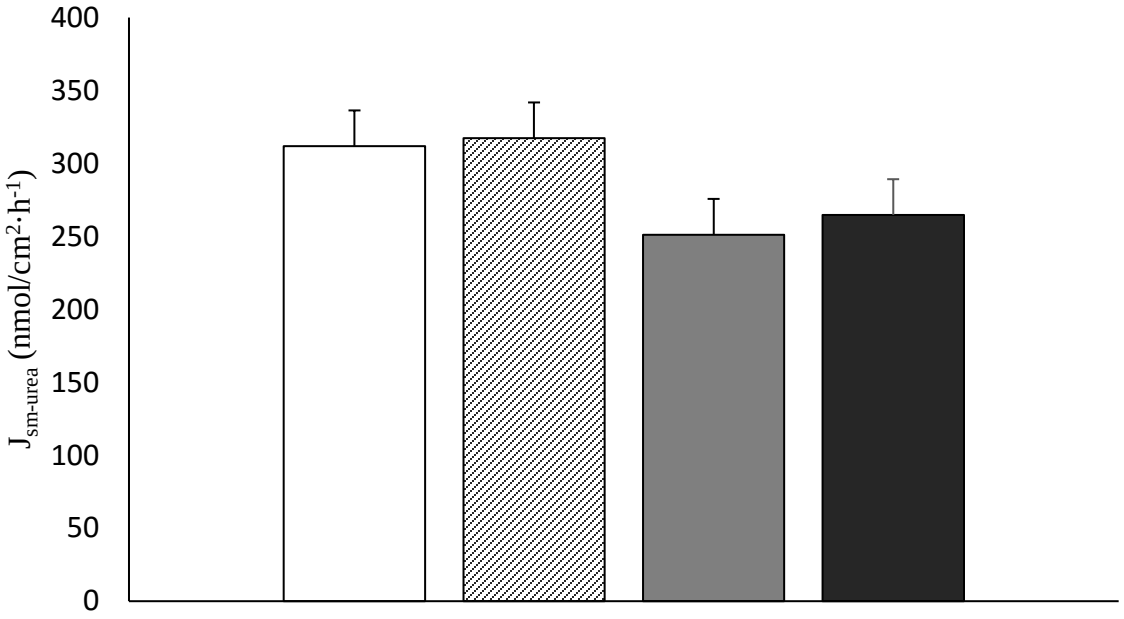

Figure 4.1. Serosal-to-mucosal flux of urea [nmol/ (cm^2 × h)] in isolated epithelia from *Bos taurus* and *Bison bison* bulls fed either a backgrounding or finishing diet. Treatments include Control (white), NH_3 (Stripes), Ni^{2+} (Light grey) and Ni^{2+} and NH_3 in combination (Dark grey).

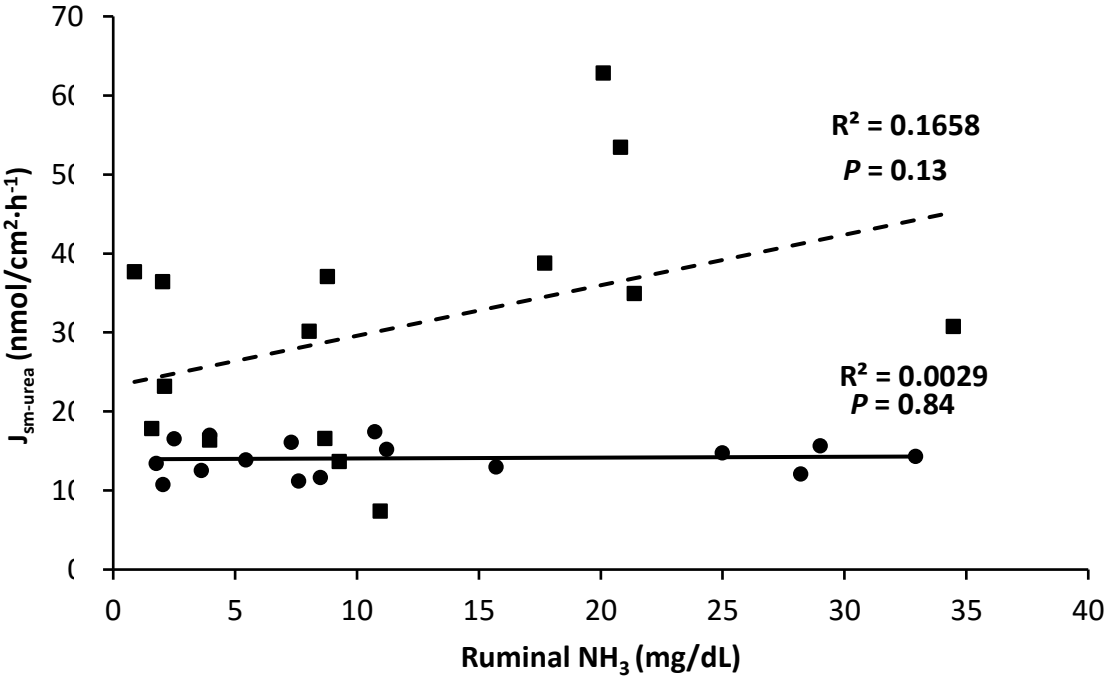

Figure 4.2. Regression analysis between ruminal NH_3 concentration at slaughter and $J_{sm\text{-}urea}$ in the isolated ruminal epithelium from *Bos taurus* (■, solid line) and *Bison bison* (●, dashed line) bulls.

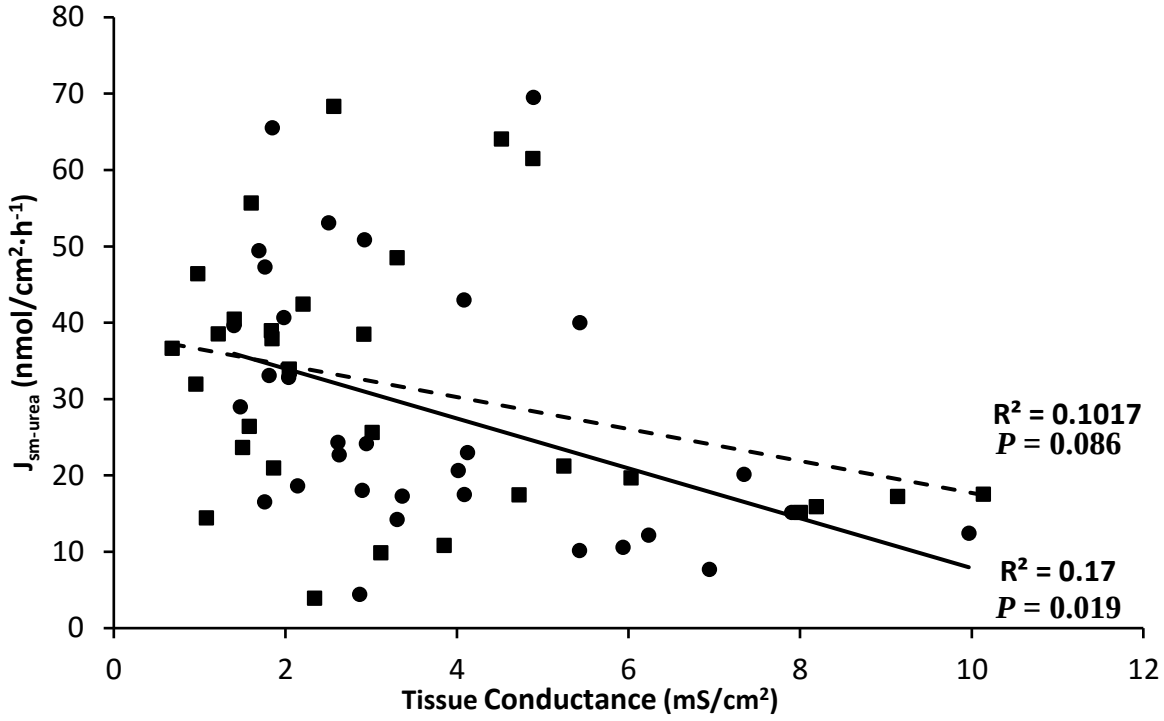

Figure 4.3. Regression analysis between tissue conductance (G_t) and $J_{sm\text{-}urea}$ in the isolated ruminal epithelium from *Bos taurus* (■, solid line) and *Bison bison* (●, dashed line) bulls.

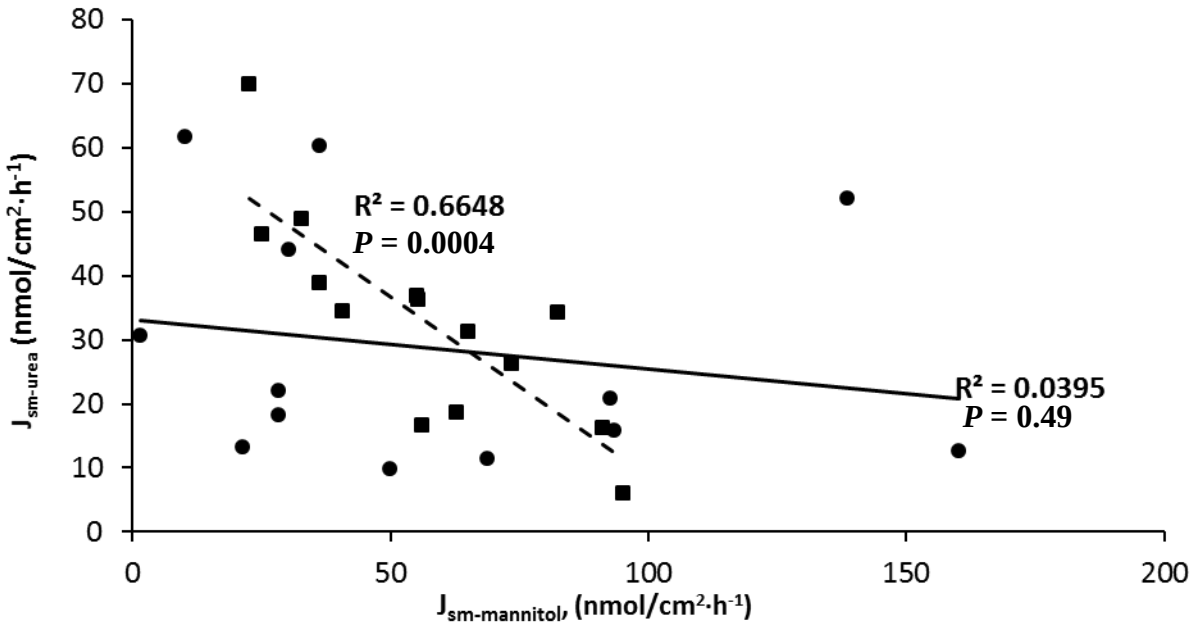

Figure 4.4. Regression analysis between $J_{sm\text{-}mannitol}$ and $J_{sm\text{-}urea}$ in the isolated ruminal epithelium from *Bos taurus* (■, solid line) and *Bison bison* (●, dashed line) bulls.

Chapter 5. INHIBITORY EFFECT OF MUCOSAL AMMONIA ON UREA FLUX ACROSS THE ISOLATED BOVINE RUMINAL EPITHELIUM IS DEPENDENT ON SEROSAL UREA CONCENTRATION

5.1. Abstract

Urea transporter-B (UT-B) proteins are involved in the movement of urea across the ruminal epithelium. Ruminal ammonia has an inhibitory effect on urea transfer from blood into the rumen, whereas urea transfer from blood into the rumen is positively correlated with blood urea-N (BUN) concentration. Because BUN concentrations typically rise in tandem with ruminal ammonia concentrations, it is of interest to determine the interactive effects of ruminal ammonia and BUN concentrations on urea movement across the ruminal epithelium. This project evaluated the effects of mucosal ammonia and serosal urea concentrations on total and UT-B-dependent (i.e., phloretin-sensitive) urea flux across the isolated bovine ruminal epithelium. Six 9-month old Holstein steers fed a common diet were slaughtered and the ruminal epithelium was collected and mounted in Ussing chambers under short-circuit conditions. To mimic physiological conditions, the serosal buffer (pH 7.4) contained 1.78 (LU) or 7.14 (HU) mmol·L^{-1} urea, whereas the mucosal buffer (pH 6.2) had no urea but contained 2.9 (LA) or 8.8 (HA) mmol·L^{-1} ammonia (added as $[NH_4]_2CO_3$). The serosal-to-mucosal flux of ^{14}C-urea ($J_{sm\text{-}urea}$) and ^{3}H-mannitol ($J_{sm\text{-}mannitol}$) were measured, with $J_{sm\text{-}mannitol}$ being used as an indicator of hydrophilic movement. Serosal addition of phloretin (1 mmol·L^{-1}) was used to inhibit UT-B-mediated urea transport. Ruminal ammonia-N and serum urea-N concentrations at slaughter averaged 7.34 and 6.32 mmol·L^{-1}, respectively. High mucosal ammonia tended to inhibit total $J_{sm\text{-}urea}$ with HU, but there was no effect of mucosal ammonia on total $J_{sm\text{-}urea}$ with LU (interaction, $P = 0.06$). The serosal addition of phloretin in the presence of serosal urea or mucosal ammonia had no effect on $J_{sm\text{-}urea}$. The $J_{sm\text{-}mannitol}$ was not affected by serosal urea ($P = 0.86$) or mucosal ammonia ($P = 0.22$) concentration. The $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ tended to be positively correlated for HA ($R^2 = 0.301$, $P = 0.08$), but not LA ($R^2 = 0.027$, $P = 0.70$) in combination with LU. The same pattern was observed with HU treatments where $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ tended to be positively correlated for HA ($R^2 = 0.329$, $P = 0.08$), but not LA ($R^2 = 0.111$, $P = 0.32$). In conclusion, mucosal ammonia has inhibitory effects on $J_{sm\text{-}urea}$ when serosal urea concentration is high, and that $J_{sm\text{-}urea}$ is mediated via hydrophilic pathways at a low serosal urea concentration but is mediated through transporter pathways that are not UT-dependent when serosal urea concentration is high.

5.2 Introduction

Domesticated ruminants, such as cattle, sheep, and goats, depend on their ability to transfer urea-N from blood to the rumen in order to maintain a positive N balance (Lapierre and Lobley, 2001). This is because endogenous urea-N production often exceeds apparent digestible N intake under a wide variety of feeding conditions and, if all endogenous urea-N output was lost in urine, it would be difficult for ruminants to maintain a positive N balance (Lapierre and Lobley, 2001). In such situations, ruminants recycle 40 to 80% of endogenously-produced urea-N to the gastrointestinal tract (GIT; Hartmeyer and Martens, 1980), particularly the rumen, where it provides ruminally-available N for the synthesis of microbial protein. Microbial protein is the major contributor to the amino acid requirements of the host animal.

Although urea-N recycling provides an opportunity to improve the efficiency of N utilization in ruminants, the regulatory mechanisms that control trans-epithelial urea-N transfer into the GIT lumen are still poorly understood (Lapierre and Lobley, 2001; Reynolds and Kristensen, 2008). Previous studies **(**Engelhardt et al., 1978; Rémond et al., 1993; Abdoun et al., 2009) have reported that increasing luminal ammonia (the sum of NH_3 and NH_4^+) concentrations caused a decrease in urea-N transfer from blood into the rumen; however, the mechanism by which ruminal NH_3-N regulates trans-epithelial urea transfer is uncertain, although elevated ruminal NH_3-N concentrations may directly reduce urea-N flux or have inhibitory effects on urease activity of epimural bacteria (Cheng and Wallace, 1979). Because the inhibitory effects of ammonia on trans-epithelial urea flux are pH-dependent (i.e., more negative as mucosal pH declines), Abdoun et al. (2009) suggested that the mechanisms involved the stimulation of the Na^+/H^+ exchanger as a means of regulating intracellular pH. However, absorption of short-chain fatty acids (SCFA) also stimulate Na^+/H^+ exchanger activity (Gäbel et al., 1991, Gäbel et al., 2002), yet SCFA promote the serosal-to-mucosal movement of urea across the isolated ruminal epithelia (Abdoun et al., 2010). Thus, the inhibitory action of ammonia remains to be elucidated. On the other hand, using the [$^{15}N_2$]-urea isotope infusion technique in sheep, Sunny et al. (2007) recently demonstrated that urea-N transfer into the GIT was positively correlated with plasma urea concentration (PUN). Because PUN largely arises from ammonia that is absorbed from the rumen (a concentration-dependent process), PUN concentrations typically rise in tandem with ruminal ammonia concentration. In such situations, it is of interest to determine how the

inhibitory effects of ruminal ammonia interact with the stimulatory effects of PUN to alter urea-N recycling.

Although passage of urea from blood into the rumen can occur via simple passive diffusion, the presence of facilitative urea transporter proteins (UT-B) in the ruminal epithelium has been demonstrated (Ritzhaupt et al., 1997). Subsequent research has demonstrated that UT-B are expressed in the stratum basale, stratum spinosum, and stratum granulosum of ruminal epithelium (Simmons et al., 2009). Proof that these UT might have a role in trans-epithelial urea flux has been provided by observations that UT-B abundance is responsive to dietary protein concentration (Marini and Van Amburgh, 2003) and forage-to-concentrate ratio (Simmons et al., 2009). Also, ex vivo work using the Ussing chamber technique showed that phloretin, a known specific inhibitor of UT-B function, reduced trans-epithelial flux of urea in isolated ruminal epithelia (Stewart et al., 2005; Abdoun et al., 2010), thus providing evidence that UT-B might play a functional role in trans-epithelial urea transfer. There is evidence that UT-B-mediated urea flux is not acutely regulated (Bagnasco, 2005; Abdoun et al., 2010), so it is unlikely that UT-B mediates the known inhibitory effects of ammonia on trans-epithelial urea flux as these are acute (Abdoun et al., 2009). However, the functional role of UT-B in mediating the effects of ruminal ammonia on trans-epithelial flux of urea remains obscure.

The objectives of this study were: 1) to determine possible interactive effects of mucosal ammonia and serosal urea concentrations on the serosal-to-mucosal urea flux across the isolated bovine ruminal epithelium; and 2) to establish if the inhibitory effects of mucosal ammonia and the stimulatory effects of serosal urea were mediated via a phloretin-sensitive pathway. Because mucosal ammonia and serosal urea have opposite effects on the permeability of the ruminal wall to urea, my hypothesis was 2-fold: 1) that there were interactive effects of mucosal ammonia and serosal urea on urea transfer across the ruminal urea; and 2) that the inhibitory effects of mucosal ammonia on urea transfer across the ruminal wall are mediated via a phloretin-sensitive pathway.

5.3 Materials and Methods

5.3.1 Animals

Six 9-month old Holstein steers were fed once daily (0730 h) for ad libitum intake. All calves received the same diet (14.2% crude protein) consisting of 38% alfalfa hay, 31% barley silage, 20% rolled barley, and 11% protein-mineral supplement (DM basis). The standard diet

was fed for a minimum of 28 d prior to the experiment and steers had free access to water. Steers were housed in the Livestock Research Building at the University of Saskatchewan, and their use in this experiment was pre-approved by the University of Saskatchewan Animal Care Committee (UCACS Protocol No. 20040066).

5.3.2 Flux measurements in Ussing Chambers

Steers were humanely killed (one per day) at 1000 h (2.5 h post-feeding) by captive bolt stunning, followed by pithing and exsanguination. Within 2 to 3 min of exsanguination, the entire digestive tract was removed from the abdominal cavity and a ~300-cm^2 piece of ruminal tissue was taken from the dorsal sac, washed in a physiological buffer solution (38°C, pH = 7.4) and gently stripped of the muscle layer. The physiological buffer solution contained ($mmol \cdot L^{-1}$): 1 $CaCl_2 \cdot 2H_2O$, 1 $MgCl_2 \cdot 6H_2O$, 2 $Na2HPO_4 \cdot 2H_2O$, 1 $NaH_2PO_4 \cdot H_2O$, 1 phenyl-phosphorodiamidate (urease inhibitor; ABCR), 5 butyric acid, 60 NaCl, 5 KCl, 10 glucose, 25 Na-acetate$\cdot 3H_2O$, 15 Na-gluconate, 10 Na-propionate, and 25 $NaHCO_3$. After stripping, the ruminal epithelium was then placed in the physiological buffer solution described above and transported to the laboratory (~5 minutes). The physiological buffer was continuously gassed with carbogen (95% O_2/5% CO_2) during tissue collection and transport.

Upon arrival at the laboratory, ruminal epithelia were cut into strips and placed between two halves of an Ussing chamber with an exposed surface area of 3.14 cm^2 as described in Chapter 2. Ruminal epithelia were exposed to 15 mL of serosal and mucosal buffer solutions. Buffer solutions were identical to the physiological buffer solution described above but also contained penicillin G sodium salt (60 $mg \cdot L^{-1}$), kanamycin sulphate (100 $mg \cdot L^{-1}$), and flurocytosine (50 $mg \cdot L^{-1}$) to inhibit microbial activity. To mimic in vivo physiological conditions, serosal and mucosal buffer solutions were adjusted to pH 7.4 and 6.4, respectively, using 1 $mol \cdot L^{-1}$ NaOH or 1 $mol \cdot L^{-1}$ HCl. A mucosal pH of 6.4 was chosen because 1) it promotes maximal trans-epithelial flux of urea in the presence of SCFA (Abdoun et al., 2010; Chapter 2), and 2) inhibitory effects of mucosal ammonia on trans-epithelial flux of urea occur at that pH (Abdoun et al., 2009). Serosal and mucosal buffer solutions were mixed using gas lift (95% O_2/5% CO_2) and were maintained at 38°C using water-jacket reservoirs. A computer-controlled voltage-clamp was used to maintain the ruminal epithelia under short-circuit conditions. Trans-epithelial conductance (G_t) was measured every 6 sec via KCl-agar bridges and Argental reference electrodes (Mettler Toledo, Urdort, Switzerland). Short-circuit current (I_{sc}) was

measured according to Aschenbach et al. (2000). Measurements of G_t and I_{sc} were averaged for each flux period according to Doranalli et al. (2011).

On each d of flux measurements, 20 min was allowed for stabilization of electrophysiology before the addition of radioisotope. The in vitro treatments were arranged in a 2 × 2 factorial arrangement with the main factors being serosal urea and mucosal ammonia concentrations. The serosal urea concentrations were 1.78 (low urea; LU) or 7.14 (high urea; HU) mmol·L^{-1}, and the mucosal ammonia (added as $[NH_4]_2CO_3$) concentrations were 2.9 (low ammonia; LA) or 8.8 (high ammonia; HA) mmol·L^{-1}. It should be acknowledged that at a mucosal pH of 6.4, more than 99% of the ammonia would be present as NH_4^+, rather than as NH_3. The ex vivo concentrations of urea and ammonia were selected to cover a range of PUN and ruminal ammonia concentrations that are typically observed in ruminants under a wide range of dietary conditions. The mucosal buffer did not contain urea. All treatments were tested with or without the serosal addition of 1 mmol·L^{-1} phloretin, a known UT-B inhibitor (Stewart et al., 2005; Abdoun et al., 2010), for a total of 8 treatment combinations. Phloretin addition was used to partition the serosal-to-mucosal urea flux ($J_{sm\text{-}urea}$) into phloretin-sensitive (i.e. likely mediated via UT-B) and phloretin-insensitive pathways. Mounted tissues were randomly assigned to one of these 8 treatments with 2 replications per treatment. All chambers contained 1 mmol·L^{-1} mannitol on the serosal side to create a serosal-to-mucosal mannitol gradient. For the measurement of $J_{sm\text{-}urea}$ and mannitol ($J_{sm\text{-}mannitol}$) fluxes, 46.25 kBq of ^{14}C-urea and 74 kBq of D-[1-^{3}H (N)]-mannitol were added to the serosal buffer, with $J_{sm\text{-}mannitol}$ being used as an indicator of hydrophilic movement. After a 45-min equilibration period, phloretin and ammonia were added to the respective chambers and allowed to mix for 5 min before the beginning of the first of two 30-min flux periods. Samples from the 'hot' (serosal side; 100 μL) and 'cold' (mucosal; 500 μL) were collected at the start and end of the flux periods. An equivalent amount of buffer was replaced after each 'cold' sample to maintain hydrostatic pressure, and this dilution was included in the flux calculations. All "cold" and "hot" samples were placed in 7-mL scintillation vials, thoroughly mixed with 5 mL of a liquid scintillation cocktail (ScintiVerse Vio-HP Cocktail, Fisher Scientific, Toronto, ON) and the radioactivity in ^{14}C-urea and ^{3}H-mannitol was determined using a liquid scintillation counter (model LS6500, Beckman Coulter, Brea, CA) in parallel. All chemicals (reagent grade), phloretin, and antibiotics were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

5.3.3 Collection and analysis of blood and ruminal fluid

Blood samples were collected from each steer into 6-mL EDTA-coated vacutainer® tubes (Becton Dickinson) 5 min prior to slaughter. Blood samples were placed on ice until being centrifuged at 3,000 x *g* for 15 minutes at 4ºC. Serum was harvested and frozen at -20ºC until being analyzed for serum urea-N (SUN) by the diacetyl monoxime method (Marsh et al., 1957) using a commercial kit (Stanbio Urea Nitrogen Kit, Procedure No. 0580; Stanbio Laboratory, Boerne, TX). Ruminal contents were collected at slaughter from the caudal-dorsal sac and strained through 2 layers of cheesecloth to obtain ruminal fluid. Ruminal pH was measured immediately using a hand-held pH meter (AP110 portable pH meter, Fisher Scientific, Toronto, ON). Thereafter, two 10-mL samples were collected and preserved with either 2 mL of metaphosphoric acid (25% wt. /v) for SCFA analysis or 2 mL of 1% sulfuric acid for ammonia analysis. Samples were stored at -20ºC. Concentrations of SCFA were determined using gas chromatography (Erwin et al., 1961). Concentration of ruminal ammonia was determined using a phenol-hypochlorite assay (Broderick and Kang, 1980).

5.3.4 Calculations and statistical analysis

Total $J_{sm\text{-}urea}$ and total $J_{sm\text{-}mannitol}$ are flux measurements that were determined from chambers that had no phloretin added to the incubation buffer. Phloretin-sensitive $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ were calculated as the difference between total J_{sm} and the J_{sm} measured in the presence of phloretin (i.e., phloretin-insensitive J_{sm}). All flux and electrophysiology data were analyzed using the mixed procedure (SAS, 2004), with flux period and treatment being considered as fixed effects, and tissue replicate within steer as a random effect. Studentized residuals were used to determine outliers with an absolute threshold value of 2. Regression analysis was performed using the regression procedure of SAS (SAS, 2004). The effect of the addition of phloretin on $J_{sm\text{-}urea}$ was tested by comparing total $J_{sm\text{-}urea}$ and phloretin-insensitive $J_{sm\text{-}urea}$ using the paired Student's *t*-test (SAS, 2004). Significance was declared when $P \leq 0.05$ and trends were considered when $0.05 < P < 0.10$.

5.4 Results and discussion

Concentrations of ruminal SCFA and ammonia, and SUN are presented in Table 5.1. Concentrations of ruminal ammonia and SUN ranged from 4.6 to 10.2 $mmol \cdot L^{-1}$ and 5.8 to 7.1 $mmol \cdot L^{-1}$, respectively, and were within the normal physiological range for ruminants. This further confirms that the range of mucosal ammonia and serosal urea concentrations that were

selected for the Ussing chamber incubations represents a wide range within values observed in cattle. Ruminal SCFA concentration averaged 129.8 mmol·L^{-1}, indicating that ruminal fermentation was active when steers were killed.

It is well-established that urea recycling to the rumen is an important evolutionary adaptation for ruminants, since ruminal bacteria are able to use recycled urea-N for the synthesis of microbial protein which, in turn, is a major contributor to the metabolizable protein supply for the host animal (Lapierre and Lobley, 2001). Urea transfer to the rumen can occur via saliva or directly across the ruminal wall. Estimates indicate that salivary transfer contributes 17 to 36% of total urea transfer depending on dietary conditions, which indicates that direct transfer across the ruminal wall is the predominant route (Lapierre and Lobley, 2001). While passive permeation of urea likely facilitates a portion of the trans-epithelial urea flux (Abdoun et al., 2010), recent studies have clearly proven that inhibition of UT-B with phloretin decreases the serosal-to-mucosal flux of urea across the isolated ruminal epithelium (Stewart et al., 2005; Abdoun et al., 2010; Doranalli et al., 2011). In addition, studies using in vivo (Rémond et al., 1993) and ex vivo (Doranalli et al., 2011) models have provided evidence suggesting there may be acute regulation of urea transfer, possibly triggered by changes in the concentrations of the end-products arising from ruminal fermentation or concentrations of blood metabolites. Indeed, increasing the intra-ruminal ammonia concentration has an inhibitory effect on trans-epithelial urea flux (Engelhardt et al., 1978; Rémond et al., 1993), whereas increasing the PUN concentration has a stimulatory effect on trans-epithelial urea flux (Sunny et al., 2007). However, it is not clear whether the inhibitory effects of phloretin (i.e., UT-B-mediated) and ammonia on urea flux are additive and whether there are interactions between the inhibitory effect of mucosal ammonia and the stimulatory effect of serosal urea. It could be speculated that the inhibitory effect of ammonia on trans-epithelial urea flux would be additive to that of phloretin since ammonia inhibition would likely represent an acute regulation of urea flux, whereas UT-B-mediated flux does not appear to be regulated acutely (Bagnasco, 2005; Abdoun et al., 2010).

Using the Ussing chamber technique, I observed that high mucosal ammonia concentration tended to inhibit total $J_{sm\text{-}urea}$ with HU, but there was no effect of mucosal ammonia on total $J_{sm\text{-}urea}$ with LU (interaction, $P = 0.03$; Table 5.2). To mimic physiological

conditions, in vitro $J_{sm\text{-}urea}$ measurements were conducted using a mucosal pH of 6.4, which has been reported to be the optimal pH for trans-epithelial urea transfer in the presence of SCFA (Abdoun et al., 2010; Chapter 2). Although previous studies demonstrated that mucosal ammonia has inhibitory effects on trans-epithelial urea transfer (Engelhardt et al., 1978; Rémond et al., 1993), my results show that these inhibitory effects are likely dependent on serosal urea concentration. Additionally, I had hypothesized that the effects of phloretin and ammonia on trans-epithelial urea flux would be additive, but my findings do not support this hypothesis. In fact, in the current study, an inhibitory effect of phloretin was not observed, which differs from most previous studies (Stewart et al., 2005; Abdoun et al., 2010; Doranalli et al., 2011). However, the incubation buffers used in those studies did not include mucosal ammonia. Moreover, Muscher et al. (2010) showed that when goats were fed a diet containing a high dietary CP concentration (19% CP) that would elevate ruminal ammonia concentration, there was no inhibitory effect of phloretin on $J_{sm\text{-}urea}$, thus supporting the findings of the current study. This data is interpreted to suggest that the inhibitory effects of ammonia and phloretin overlap. However, I cannot rule out that the UT-B carrier-mediated urea transport pathway may not have been functional under the incubation conditions in this study. Also, other facilitative transporters that are expressed in ruminal epithelium and are phloretin-insensitive could be involved in $J_{sm\text{-}urea}$ trans-cellular urea fluxes at a low mucosal ammonia concentration. Potentially aquaglyceroporins, a family of membrane-spanning proteins that are predominantly involved in water transport, but are also permeable to urea and have been demonstrated to be expressed in bovine ruminal epithelium (Røjen et al., 2011a) may have been active. Since the completion of this study, Lu et al. (2014) demonstrated that ex vivo administration of 2.5 m*M* NH_3 significantly reduced $J_{ms\text{-}urea}$ and $J_{sm\text{-}urea}$ in ruminal epithelial tissue collected from growing lambs fed a complete hay diet. However, in that study the impact that mucosal NH_3 has on phloretin sensitive $J_{sm\text{-}urea}$ was observed. In the current study a significant inhibition of total $J_{sm\text{-}urea}$ through administration of a high dose of mucosal NH_3 was observed, which is in agreement with Lu et al. (2014), however; in the present study an inhibitory effect of NH_3 was not observed on phloretin sensitive $J_{sm\text{-}urea;}$ thus, it is possible that the inhibitory effects of NH_3 on $J_{sm\text{-}urea}$ are not directly inhibiting phloretin sensitive pathways.

From a physiological standpoint, this serosal urea-dependent effect of mucosal ammonia on trans-epithelial urea flux may be of practical importance in regulating N supply for microbial

growth under varying feeding conditions. Depending on dietary conditions, ruminal ammonia concentrations reach a peak within 1 to 2 h after feeding, and portal uptake of ammonia can be high at this time if ruminal energy supply is limiting (Lapierre and Lobley, 2001). Ruminal ammonia absorption into portal blood significantly contributes to endogenous urea production in the liver (Lapierre and Lobley, 2001); thus, a post-prandial elevation in serum urea concentration (SUN) concentration is typically observed in ruminants, although this might slightly lag behind ruminal ammonia peaks. High SUN is normally observed, therefore, at high intakes of dietary N or ruminally-degradable N, so ammonia requirements for microbial growth can be met from diet-derived ruminal ammonia and there is no necessity for urea recycling. On the other hand, low SUN concentrations might be reflective of a ruminal ammonia deficiency, thus signaling a greater need for urea recycling to satisfy microbial ammonia requirements.

In the present study, trans-epithelial mannitol fluxes ($J_{sm\text{-}mannitol}$) that were measured concurrently with urea fluxes ($J_{sm\text{-}urea}$) were used an indicator of hydrophilic (mainly paracellular) movement (Stewart et al., 2005; Abdoun et al., 2010). My results showed that $J_{sm\text{-}mannitol}$ was not affected by serosal urea ($P = 0.86$), or mucosal ammonia ($P = 0.22$) concentrations, as well as their interaction ($P = 0.38$; Table 5.2). This finding supports that of Abdoun et al. (2010) indicating that the trans-epithelial movement of mannitol and urea are independent and that the urea flux is likely transcellular (Abdoun et al., 2010). In order to partition trans-epithelial urea flux between paracellular- and transcellular-mediated routes, plots of $J_{sm\text{-}urea}$ against $J_{sm\text{-}mannitol}$ are presented in Figures 5.1 and 5.2. Results show that $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ tended to be positively correlated for HA ($R^2 = 0.301$, $P = 0.08$), but not for LA ($R^2 = 0.027$, $P = 0.70$), in combination with LU (Figure 5.1). The same pattern was observed with HU treatments where $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ tended to be positively correlated for HA ($R^2 = 0.329$, $P = 0.08$), but not for LA ($R^2 = 0.111$, $P = 0.32$; Figure 5.2). The tendency for a significant correlation between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ at a high mucosal ammonia concentration suggests that a proportion of trans-epithelial urea flux occurs via a hydrophilic (paracellular) pathway. At a low mucosal ammonia concentration, the lack of a significant correlation between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ indicates that a proportion of trans-epithelial urea flux occurs via a trans-cellular pathway (mediated through UT-B).

Tissue conductance (G_t) was not affected by the serosal urea concentration ($P = 0.53$) or mucosal ammonia concentration ($P = 0.76$), whereas short-circuit current (I_{sc}) was significantly increased with HA ($P = 0.006$), but was unaffected by serosal urea concentration ($P = 0.79$; Table 5.2). The increase in I_{sc} with greater mucosal ammonia concentration has been reported to be pH-dependent and attributable to the stimulation of electrogenic Na transport (Abdoun et al., 2005). Increasing the mucosal ammonia concentration (as NH_4Cl) decreased the mucosal-serosal Na flux ($J_{ms\text{-}Na}$) when the pH of the mucosal buffer was 7.4, but $J_{ms\text{-}Na}$ was increased at pH 6.4 (Abdoun et al., 2005). According to the Henderson-Hasselbalch equation, 99% of the ammonia will be in the form of NH_4^+ at pH 6.4. In the protonated form, NH_4^+ crosses the mucosal membrane via K^+ channels (Gutman et al., 2003) into the cytoplasm (pH 7.4), where a portion of the NH_4^+ dissociates into NH_3 and H^+. To avoid intracellular acidity, the free H^+ is exchanged with Na^+ via Na^+/H^+ exchangers in the apical membrane (Aronson et al., 1982), while an equimolar amount of Na^+ is excreted on the basolateral side via the Na^+/K^+-ATPase causing an increase in I_{sc} (Abdoun et al., 2005).

Table 5.1. Rumen ammonia and blood analysis.

Item	Value
Total VFA, mmol·L^{-1}	129.8
Acetate, mmol/100mmol	53.0
Propionate, mmol/100mmol	19.3
Butyrate, mmol/100mmol	18.4
Isobutyrate, mmol/100mmol	2.6
Valerate, mmol/100mmol	2.7
Isovalerate, mmol/100mmol	3.5
Caproate, mmol/100mmol	0.3
Acetate: propionate ratio	2.74
NH_3-N, mmol·L^{-1}	7.34
SUN, mmol·L^{-1}	6.32

volatile fatty acid, urea nitrogen

Table 2. Urea flux rates and electrophysiology measurements in ruminal epithelial tissue obtained from Holstein steer calves (n = 6) and incubated in Ussing chambers with varying concentrations of mucosal ammonia and serosal urea with or without phloretin

Item	Treatment				SEM	*P* values		
	High Urea		Low Urea					
	Low ammonia	High ammonia	Low ammonia	High ammonia		Urea	Ammonia	Urea x ammonia
Fluxes, nmol·$(cm^2)^{-1}$·h^{-1}								
Total $J_{sm\text{-}urea}$	771.8	679.8	168	163.7	30.4	<0.001	0.06	0.03
Phloretin-insensitive $J_{sm\text{-}urea}$	781.7	717.5	181.7	164.9	34.4	<0.001	0.10	0.06
Total $J_{sm\text{-}mannitol}$	63.6	55.6	51.1	44.9	7.3	0.86	0.22	0.38
G_t, mS·$(cm^2)^{-1}$	3.78	3.67	3.31	3.11	0.32	0.53	0.76	0.92
I_{sc}, μA	14.27	16.63	14.78	16.65	0.41	0.79	0.006	0.72

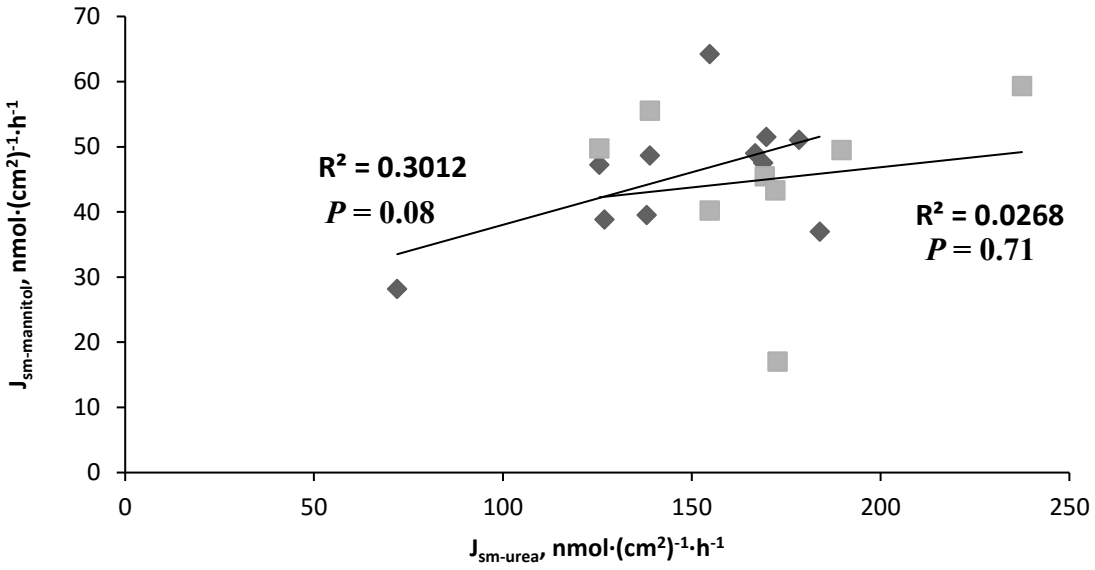

Figure 5.1. Regression analysis between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ fluxes across the ruminal epithelium in incubations containing 1.78 mmol·L^{-1} serosal urea (LU) with 2.9 (♦) or 8.8 (■) mmol·L^{-1} ammonia.

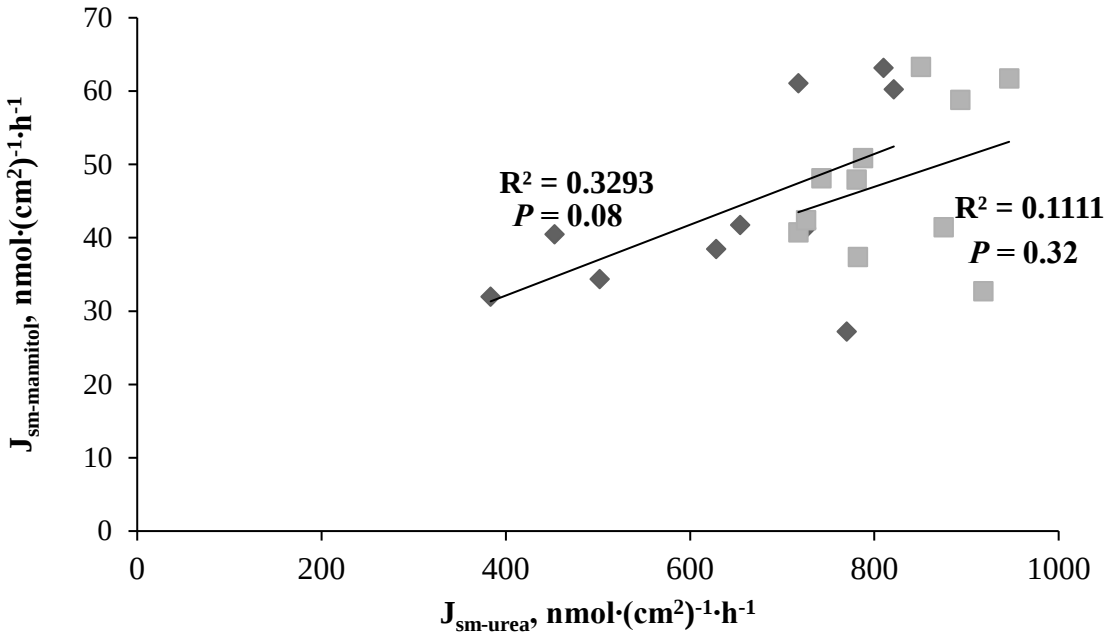

Figure 5.2. Regression analysis between $J_{sm\text{-}urea}$ and $J_{sm\text{-}mannitol}$ fluxes across the ruminal epithelium in incubations containing 7.14 mmol·L^{-1} serosal urea (HU) with 2.9 (♦) or 8.8 (■) mmol·L^{-1} ammonia.

CHAPTER 6. GENERAL DISCUSSION

Transport of urea-N across the ruminal epithelium plays a particularly large role in N salvage and return to the GIT for microbial protein synthesis in ruminants (Lapierre and Lobley, 2001), thus understanding the mechanisms responsible for trans-epithelial urea flux is important for the future adaptation of feeding strategies to improve production as well as reduce the environmental impact of livestock agriculture. Experiments are presented in this thesis with the overall objective of determining the functional roles of UT-B and AQP proteins in trans-epithelial urea transport (Chapter 2), and how their roles are affected by an abrupt change in dietary carbohydrate fermentability (Chapter 3), the effect of ammonia on AQP-mediated trans-epithelial urea flux (Chapter 4), as well as the effect of varying NH_3 and urea concentrations on UT-B mediated urea flux (Chapter 5).

Many studies have highlighted the fact that when diet fermentability is increased in ruminants, the depression in ruminal ammonia concentration that is created from the increase in microbial protein synthesis increases urea-N recycling to the GIT (Bach et al., 2005). It is well known that UT-B plays a role in this increase (Doranalli et al., 2011); however, I wanted to determine the role of AQP in relation to UT-B proteins when ruminants undergo an abrupt change in diet fermentability, similar to the dietary changes that a close-up dairy cow moving to the lactating cow ration must endure or that a beef animal undergoes when it is moved off pasture, and arrives in the receiving pen at the feedlot.. In chapter 2; I was able to determine that AQP can be inhibited through the addition of 1mmol/L$NiCl_2$. In chapter 3, results indicated that with an abrupt change in diet fermentability, $J_{sm\text{-}urea}$ increased linearly with increased days when steers were fed a moderately fermentable diet. I also determined that AQP play an important functional role in this response as there was a tendency for Ni-sensitive $J_{sm\text{-}urea}$ to increase linearly with advancing days after the abrupt change in diet fermentability occured. As well as functional adaptation, mRNA abundance of both AQP-3, a known urea transporting AQP, and UT-B linearly increased with increasing days of adaptation to the abrupt change in diet fermentability. Although other studies have demonstrated changes in mRNA abundance of AQP (Røjen et al., 2011a) as well as UT-B (Stewart et al., 2005; Muschler et al, 2010; Røjen et al., 2011a) to changing dietary conditions, to my knowledge I are the first to demonstrate the functional significance of AQP-mediated $J_{sm\text{-}urea}$ in relation to UT-B mediated $J_{sm\text{-}urea}$. This

increase in mRNA abundance of AQP proteins, along with the significant linear increase in Ni-sensitive $J_{sm\text{-}urea}$, clearly demonstrates the importance of AQP in the transport of urea across the ruminal epithelium in response to an adaption to a moderately fermentable diet.

Along with functional changes in urea kinetics, it is well documented that the ruminal epithelium undergoes significant changes in sodium and short-chain fatty acid transport mechanisms after an abrupt change in diet fermentability (Aschenbach et al., 2011). Schurmann et al. (2014) noted that when calves were abruptly switched to a moderately fermentable carbohydrate diet, that there were significant functional adaptive increases in both SCFA as well as Na^+ absorption. After witnessing the change in the relative importance of AQP mediated $J_{sm\text{-}urea}$, likely due to a depression in ruminal ammonia concentration, with significant ruminal adaptation taking place within 3 days of an abrupt change in diet fermentability, I decided to investigate the in vivo short term adaptation of AQP mediated $J_{sm\text{-}urea}$ when ruminants are fed diets containing moderately fermentable carbohydrates (Chapter 4). I also questioned whether or not the impact of moderate or highly fermentable diets would impact $J_{sm\text{-}urea}$ in bison compared with beef cattle; knowing that bison have been documented to be more efficient with their use of dietary N when compared to bovine animals (Deliberto, 1993).

In this experiment (Chapter 4) I noted that there was a tendency for an increase in total $J_{sm\text{-}urea}$ when bison and bovine animals were fed finishing diets compared to backgrounding diets; this is in agreement with Huntington and Archibeque (1999) and Bach et al. (2005) where the authors outlined that increasing microbial protein through additional dietary fermentable carbohydrates has demonstrated to increase N return to the GIT. This was similarly noted in (Theurer et al., 1999, 2002) where the authors noted an increase in N returned to the GIT by increasing the fermentability of sorghum grain through steam flaking. However, although there were no dietary and species differences noted in $J_{sm\text{-}urea}$, it was noted that again, 1 m*M* administration of $NiCl_2$ significantly reduced the transepithelial movement of urea. The extent of this inhibition was not different between species nor dietary treatment, demonstrating that AQP have a significant contribution to the total $J_{sm\text{-}urea}$. However, from this study I determined that in vivo administration of 7 m*M* NH_3 had no impact on $J_{sm\text{-}urea}$, regardless of species or dietary treatment. This was not in agreement with current theories of the impact of ruminal NH_3 on urea recycling (Lapierre and Lobley, 2001). There is limited research demonstrating the

impact of in vivo ruminal NH_3 concentration on $J_{sm\text{-}urea;}$ however, Lu et al. (2014) noted that when 2.5 m*M* NH_4Cl was added *in vitro* to isolated ruminal epithelia from lambs fed a strictly hay diet, that $J_{sm\text{-}urea}$ was reduced, when mucosal pH was maintained at 6.4, but was not affected when pH was at 7.4. This major difference may be due to the lack of fermentable carbohydrates offered to the lambs in the study by Lu et al. (2014)

As described in Chapter 4, Na^+ absorption across the ruminal epithelium in exchange for H^+ via sodium-hydrogen exchangers is a mechanism for intracellular pH regulation (Gäbel et al., 1989; Gäbel et al., 1991; Sehested et al., 1999; Etchmann et al., 2009; Shen et al., 2012). Abdoun et al. (2009) demonstrated that under Ussing chamber conditions, when the isolated ruminal epithelium is exposed to SCFA that there is an increase in the epithelial serosal to mucosal movement of urea. In this way, I surmised that Na^+ transport may be used as an indicator for SCFA absorption in addition to the potential effect that NH_4^+ may have on Na^+ transport (Lu et al., 2015) (Chapter 4). Schurmann et al. (2014) noted that SCFA absorption across the ruminal epithelial membrane was still occurring to 21 days after an abrupt change in dietary carbohydrate fermentability, with $J_{net\text{-}Na}$ peaking after 7 days of adaptation, and decreasing thereafter. Results in Chapter 4 revealed that there was a lack of dietary effect on the flux of Na^+ across the ruminal epithelium, with ruminal SCFA concentrations not different between animals fed moderate or highly fermentable carbohydrate diets. It would appear that since bulls were on their dietary treatments for a minimum of 30 days, that ruminal adaptation to their respective diets had occurred. Lu et al. (2014) proposed that the decrease in $J_{sm\text{-}urea}$ in the presence of NH_3 was in part due to a ruminal epithelial cell attempting to regulate intracellular pH. Recent work has demonstrated that both AQP and UT-B are sensitive to changes in pH (Sands, 2003; MacIver et al., 2009), potentially accounting for this depression in $J_{sm\text{-}urea}$ (Lu et al., 2014) that was observed in Chapter 5. In this experiment, an argument can be made that both bison and beef bulls had sufficient time to adapt to the dietary treatments, thereby preventing a depression in $J_{sm\text{-}urea}$, due to a depression in ruminal pH caused by the high rate of microbial carbohydrate fermentation when ruminants are fed moderate and highly fermentable carbohydrate. This high rate of microbial protein synthesis due to the available carbohydrate sources utilizes significant amounts of ruminal NH_3. Once harvested this ruminal epithelium is unaccustomed to a physiologically high level of mucosal NH_3 in vivo, and thus it is plausible that the lack of ex vivo NH_3 inhibition on $J_{sm\text{-}urea}$ is due to the lack of ruminal epithelial

adaptation to high ruminal NH_3. The inverse of this relationship has been demonstrated multiple times (Stewart et al., 2005; Muschler et al., 2010; Doranalli et al., 2011), where ruminal NH_3 concentration measured in *vitro* is negatively correlated with $J_{sm\text{-}urea}$ ex vivo.

This negative relationship between ex vivo mucosal NH_3 concentration and $J_{sm\text{-}urea}$ was noted in Chapter 5 where, regardless of ex vivo mucosal NH_3 concentration, but was only seen when serosal urea concentration was at 7.14 m*M*. Although dietary crude protein of the experimental diets was similar for all previous chapters, the current study utilized a high level of alfalfa hay, known to have a higher CP degradability in the rumen than barley silage or grass hay (NRC, 2001). However, in this study there was a tendency for a mucosal NH_3 concentration of 8.8 m*M* to reduce $J_{sm\text{-}urea}$ compared with tissues incubated with mucosal NH_3 concentrations of 2.9 m*M* at the high serosal urea concentration, but was not observed at a low serosal urea. During this study, the goal was to mimic the daily, acute changes to the ruminal epithelium, where animals undergo periods of high/low ruminal NH_3 in combination with varying high/low serum urea concentrations. This is especially true when animals are fed oscillating protein diets, where animals will be fed N deficient diets for days at a time, followed by an overfeeding of digestible N (Archibeque et al., 2007). Adaptation to a diet that creates a ruminal environment that is high in NH_3, and lacks the available carbohydrates to create an osmotic drive for the trans-epithelial movement of serosal urea, leaving me to hypothesize that the ruminal epithelium will possess a stronger ability to reduce $J_{sm\text{-}urea}$ in the presence of mucosal NH_3. Increased $J_{net\text{-}Na}$ in response to an increase in ruminally available SCFA, resulting in an increase in SCFA absorption, has been described as a coping mechanism for maintaining intracellular pH (Gäbel et al., 1989; Gäbel et al., 1999; Aschenbach et al., 2011). Similarly to H-SCFA dissociation to SCFA and H^+ after being absorbed into the cytosol, Lu et al. (2014) proposed that NH_4^+, the predominant form of NH_3 at physiologic rumen conditions, acts much in the same way as H-SCFA. Upon entering the cytosol, NH_4^+ is dissociated to NH_3 and H^+, with NH_3 being absorbed into the blood stream and transported to the liver for detoxification to urea. Through the action of sodium hydrogen exchanger (NHE), the excess H^+ ions are translocated out of the cytosol in exchange for Na^+ from the rumen, thereby stabilizing the cytosolic pH. However, since NH_4^+ is the predominant form of NH_3 in the rumen, and NH_4^+ poorly diffuses across lipid membranes, thus it is plausible that NH_4^+ is predominantly transported via facilitative transport mechanisms to the blood. Urea transport proteins such as UT-B have been documented to transport NH_3 in

other species (Sands, 2003; Weihrauch et al., 2008; Braun and Perry, 2010), and AQP have the ability to transport NH3, glycerol, and protons (Beitz et al., 2005; Litman, et al., 2009). Thus, it is plausible that the drop in cytosolic pH that is caused by an influx of NH_4^+, causes an inhibition in the functional properties of AQP and UT-B, thereby reducing the capacity for the facilitative transport of urea. In the future, determining the mechanism for the inhibitory effect of NH_3 on $J_{sm\text{-}urea}$ will need to be further investigated, as well as the impact of diet on the NH_3 sensitive portions of $J_{sm\text{-}urea}$.

In closing, this thesis provides many findings in the mechanisms involved in trans-epithelial movement of urea. This research provides evidence that both AQP and UT-B play an important role in the trans-epithelial movement of urea, and that their role is important in the adaptive increase in $J_{sm\text{-}urea}$ when ruminants are fed diets containing moderate levels of fermentable carbohydrates. I also determined that when beef cattle are fed moderate and highly fermentable diets, that NH_3 had little impact on AQP-mediated and total $J_{sm\text{-}urea}$. This was also found in bison fed the same diets. Finally, I noted that when exposed to varying levels of mucosal NH_3, there is an inhibitory effect of NH_3 on $J_{sm\text{-}urea}$; however, in the Chapter 5 I did not detect an inhibitory effect on phloretin- or UT-B-mediated $J_{sm\text{-}urea}$. In the future, additional research will be required to determine the mechanisms involved in NH_3 inhibition of $J_{sm\text{-}urea}$, as this key step is critical in the role of urea-N recycling in ruminants.

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